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# Correlation of structure and function in the $Ca^{2+}$-ATPase of sarcoplasmic reticulum: a Fourier transform infrared spectroscopy (FTIR) study on the effects of dimethyl sulfoxide and urea

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**The effect of dimethyl sulfoxide (DMSO) on the structure of sarcoplasmic reticulum was analyzed by Fourier transform infrared (FTIR) and fluorescence spectroscopy. Exposure of sarcoplasmic reticulum vesicles to 35% DMSO (v/v) at 2°C for several hours in a $D_2O$ medium produced no significant change in the phospholipid and protein Amide I regions of the FTIR spectra, but the intensity of the Amide II band decreased, presumably due to proton/deuterium exchange. At 40% to 60% DMSO concentration a shoulder appeared in the FTIR spectra at 1630 $cm^{-1}$, that is attributed to the formation of new $\beta$ or random coil structures; irreversible loss of ATPase activity accompanied this change. At 70% DMSO concentration the intensity of the main Amide I band at 1639 $cm^{-1}$ decreased and a new band appeared at 1622 $cm^{-1}$, together with a shoulder at 1682 $cm^{-1}$. These changes indicate an abrupt shift in the conformational equilibrium of $Ca^{2+}$-ATPase from $\alpha$ to $\beta$ structure or to a new structure characterized by weaker hydrogen bonding. Decrease of ionization of aspartate and glutamate carboxyl groups in the presence of DMSO may also contribute to the change in intensity at 1622 $cm^{-1}$. The changes were partially reversed upon removal of DMSO. Exposure of sarcoplasmic reticulum vesicles to 1.5 kbar pressure for 1 h at 2°C in an EGTA-containing (low $Ca^{2+}$) medium causes irreversible loss of ATPase activity, with the appearance of new $\beta$ structure, and abolition of the $Ca^{2+}$-induced fluorescence response of FITC covalently bound to the $Ca^{2+}$-ATPase; DMSO (35%) stabilized the $Ca^{2+}$-ATPase against pressure-induced changes in structure and enzymatic activity, while urea (0.8 M) had the opposite effect.**

## Introduction

The kinetic mechanism of ATP-dependent $Ca^{2+}$ translocation by the $Ca^{2+}$-ATPase has been developed in considerable detail [1–5]. Much information is also available on the primary sequence [6–8] and overall shape [9–16] of the $Ca^{2+}$-ATPase, but the secondary and tertiary structure of the enzyme is as yet largely undefined. The conformational transitions proposed on the basis of kinetic studies [1,2] are supported by fluorescence [17–24], partial proteolysis [10,25,26] and crystallographic [10,11,14–16] data. However, circular dichroism studies detected no significant changes in secondary structure during transition between the two major conformations of the $Ca^{2+}$-ATPase [27,28], and only slight differences were seen by FTIR spectroscopy [29]. Based on these observations, the transition between the $E_1$ and $E_2$ states of $Ca^{2+}$-ATPase is probably accomplished by relative sliding or hinge-like motions of domains, without large-scale changes in the secondary structure. It is clear from these considerations that further advance in the development of the molecular mechanism of $Ca^{2+}$ translocation requires more structural data.

Dimethyl sulfoxide reversibly inhibits the ATPase activity of sarcoplasmic reticulum [30]. The inhibition is nearly complete at a DMSO concentration of about

Abbreviations: $Ca^{2+}$-ATPase, $Ca^{2+}$-transporting ATPase (EC 3.6.1.38); DMSO, dimethyl sulfoxide; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; FITC, fluorescein-5'-isothiocyanate; FTIR, Fourier transform infrared.

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40%, but full activity can be regained after dilution of DMSO to 4% or less. The phosphorylation of the $Ca^{2+}$-ATPase by inorganic phosphate was facilitated by 20–40% DMSO, but synthesis of ATP from the phosphoenzyme was observed only at low DMSO concentration [31]. The effect of DMSO on the partial reactions of the $Ca^{2+}$-ATPase was counteracted by urea [32,33].

In this report we analyze the effects of DMSO, urea and high pressure on the structure of $Ca^{2+}$-ATPase using Fourier transform infrared and fluorescence spectroscopy, and correlate the observed changes with effects on the steady state rate of ATP hydrolysis.

## Experimental procedures

### *Materials*

Adenosine 5′-triphosphate, EGTA, imidazole, NADH, magnesium chloride, lactate dehydrogenase (rabbit muscle), pyruvate kinase (rabbit muscle), phospho*enol*pyruvate, bovine serum albumin, dimethyl sulfoxide and Trizma base were obtained from Sigma Chemical Co., St. Louis, MO 63178. Sodium vanadate was the product of Fisher Scientific Co., Fairlawn, NJ 07410. Calcium chloride was obtained from Baker Chemical Co., Phillipsburg, NJ 08854; A23187 from Behring Diagnostics, La Jolla, CA 92037; FITC from Molecular Probes, Inc., Eugene OR 97402; $D_2O$ (99.9%) from MDS Isotope, Division of Merck Frosst, Canada, Inc., Montreal, Canada. All chemicals were of analytical grade.

### *Methods*

*(a) Isolation of sarcoplasmic reticulum.* Sarcoplasmic reticulum vesicles were isolated from rabbit muscles according to Nakamura et al. [34]. 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°C.

*(b) Preparation of microsomes for FTIR measurements.* Before pressure treatment, 4-ml aliquots of microsome suspensions were thawed, diluted 6-times with $D_2O$ medium containing 0.1 M KCl, 1 mM $MgCl_2$, and 10 mM imidazole, pD 7.4 (pH 7.0, according to Glasoe and Long [35]). The vesicles were centrifuged for 30 min at $92\,000 \times g$; the pellets were resuspended in the same medium and centrifugations were repeated twice more. The final sediments were taken up in $D_2O$ medium to a final protein concentration of 40–65 mg/ml. A similar procedure was used for the preparation of microsomes in $H_2O$ medium. Protein concentration was determined according to Lowry et al. [36].

Additions of $Ca^{2+}$, EGTA and vanadate were made from $D_2O$- or $H_2O$-containing stock solutions to final concentrations indicated in the legends. DMSO, in final concentrations indicated in the text, was added drop by drop while vortexing strongly the microsome preparation.

*(c) Pressure treatment.* For pressure treatment, glass vials containing 300 μl microsomes were capped by three layers of parafilm and were placed in an SLM-Aminco French pressure cell (Model FA-030, 1-inch diameter piston, 35 ml capacity, rated at 2.72 kbar). Pressure was applied in the range of 1 bar to 2 kbar in the SLM-Aminco French press (Model FA-078) at 2–4°C for periods ranging from 1 to 2 h. Aliquots of the same solutions were kept at 1 bar as controls.

*(d) FTIR measurements.* Aliquots (10 μl) taken from the control and pressure treated sarcoplasmic reticulum samples were used for parallel measurements of FTIR spectra in a Mattson Cygnus 100 spectrometer equipped with barium fluoride windows and 50 μm spacers for samples in $D_2O$ medium and 12 μm spacers for samples in $H_2O$ medium. Temperature controlled cells were used (Model TFC-M25, Harrick Scientific Corp., Ossining, NY 10562) with circulating water bath (Brinkmann model RM6). The temperature was measured with a thermocouple placed close to the cell windows and all FTIR measurements were made at 20°C. 256 scans were collected, coadded, zero filled one time, and apodized with triangular function. The resolution was 2 $cm^{-1}$.

The use of vibrational spectroscopy for probing the structure of proteins in biological membranes is well documented [37–40]. Recent developments in instrumentation and in data analysis have significantly improved the resolution of the broad multicomponent Amide I and Amide II bands in the infrared spectra of proteins [40–44]. Among the various resolution enhancement procedures we utilized deconvolution [41,42,44] and difference spectra [37] for the separation and interpretation of overlapping component bands.

*(e) ATPase activity measurements.* ATPase activities were measured by a coupled enzyme system of pyruvate kinase and lactate dehydrogenase [45,46]. Medium I contained 0.1 M KCl, 0.02 M Tris-HCl (pH 7.5), 1.4 mM $CaCl_2$, 5 mM $MgCl_2$, 0.42 mM phospho*enol*-pyruvate, 0.2 mM NADH, 7.5 IU of pyruvate kinase, 18 IU of lactate dehydrogenase and 2 μM A23187.

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, 0.42 mM phospho*enol*pyruvate, 0.2 mM NADH, 7.5 IU of pyruvate kinase and 18 IU of lactate dehydrogenase.

Immediately after pressure treatment, 10-μl aliquots of control or pressure treated samples were diluted with $H_2O$ medium to a final protein concentration of 1 mg/ml. Then 10-μl aliquots of the diluted samples were added to 1 ml medium I (microsomal protein concentration, 10 μg/ml), and the reaction was started after incubation for 5 min at 25°C by addition of 1 ml of medium II. The ATPase activity was measured in an Aminco DW-2 spectrometer at 340 nm at 25°C. For

measurements of the $Ca^{2+}$-insensitive ATP hydrolysis. $Ca^{2+}$ was omitted from the medium. The ATPase activity data were corrected for the $Ca^{2+}$-insensitive hydrolysis rate.

*(f) Polarization of fluorescence of FITC-labeled sarcoplasmic reticulum.* Sarcoplasmic reticulum vesicles (2 mg protein/ml) were labeled with FITC at a concentration of 5 nmoles/mg protein, essentially as described earlier [47]. The polarization of FITC fluorescence was measured at excitation and emission wavelengths of 490 and 525 nm, respectively, in an SLM 4800 fluorescence spectrometer equipped with Glan-Thompson polarizers, at 25° C. Each polarization value represents the average of 400 intensity readings, with corresponding standard deviations. The protein concentration was 50 $\mu$g/ml.

## Results

### *The effects of dimethyl sulfoxide on the FTIR spectrum of sarcoplasmic reticulum*

The FTIR spectrum of sarcoplasmic reticulum (SR) in a $D_2O$ medium containing 0.1 M KCl, 10 mM imidazole (pD 7.4), 1 mM $MgCl_2$, and $10^{-5}$ M $Ca^{2+}$ is shown in Figs. 1 and 2A. The broad band centered at 1728 $cm^{-1}$ corresponds to the carbonyl groups of the phospholipids. The intense peak at 1645 $cm^{-1}$ is the Amide I band, which arises mainly from the carbonyl stretching vibrations of the protein. The Amide II band located at 1548 $cm^{-1}$ represents a mixture of in-plane deformations of NH groups, and of stretching vibrations of CN groups within the protein. The broad peak at 1575 $cm^{-1}$ contains vibrations of protein side chains.

The spectra of SR taken in the absence of DMSO and in the presence of 35% DMSO (Fig. 1) show no significant differences in the phospholipid (1728 $cm^{-1}$) and Amide I (1645 $cm^{-1}$) regions. The decrease in the intensity of 1548 $cm^{-1}$ band in the presence of 35% DMSO was evident even after subtraction of the solvent background, and it is attributed to enhanced proton-deuterium exchange in the NH groups of proteins. Since the SR vesicles had been washed repeatedly with $D_2O$ medium prior to the measurements, permitting the exchange of accessible protons, the residual Amide II band observed in the absence of DMSO corresponds to the NH groups that are poorly accessible to water. The increased H-D exchange in the presence of DMSO suggests increased contact between water and these regions of the protein. Otherwise the differences seen in the 1600 $cm^{-1}$ to 1500 $cm^{-1}$ range are mainly due to differences in the solvent background. The intensity of the band due to HDO deformation-stretching at 1450 $cm^{-1}$ decreases with increasing concentration of DMSO,

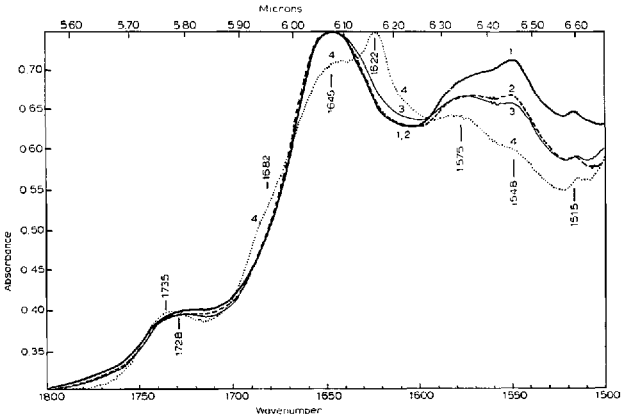


Fig. 1. The effect of DMSO on the FTIR spectra of sarcoplasmic reticulum in $D_2O$ medium. The IR spectrum of SR vesicles (20 mg/ml protein) was measured in $D_2O$ medium containing 0.1 M KCl, 10 mM imidazole (pD 7.4), 1 mM $MgCl_2$, $10^{-5}$ M $Ca^{2+}$ without DMSO (line 1, ———), and DMSO at concentrations of 35% (line 2, – – –), 45% (line 3, ———) and 70% (line 4, · · · · · ·). The spectra have not been corrected for solvent, but water vapors were subtracted. The path length was 50 $\mu$m and windows were $BaF_2$.

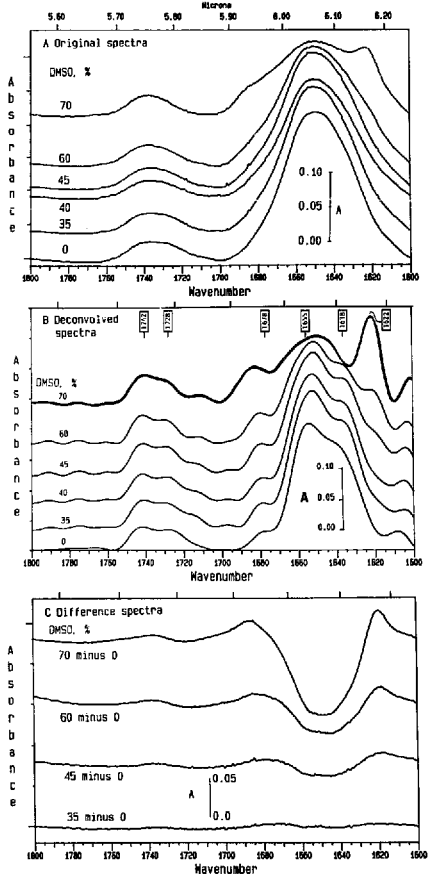


Fig. 2. Original, deconvolved and difference spectra of sarcoplasmic reticulum at various concentrations of DMSO. The spectra of sarcoplasmic reticulum taken at various DMSO concentrations were measured as described in Fig. 1. The spectra were baseline corrected and water vapor subtracted. The $\lambda$. fPase activities of these samples are given in Table I. Panel A: Original spectra. Panel B: Same spectra after deconvolution using 25 $cm^{-1}$ Lorentzian bandwidth and 1.8 resolution factor. Panel C: Difference spectra between the original spectra of sarcoplasmic reticulum (panel A) with and without DMSO.

causing a progressive diminution of the background spectrum.

The deconvolution technique facilitates the resolution of finer features of the FTIR spectrum [40–44]. The resolution enhancement is achieved at the expense of some distortion of the original band shape [43]. The choices of deconvolution parameters such as the Lorentzian bandwidth and the resolution factor determine the extent of resolution of subcomponents under the composite band. The conservative choice of these parameters is imperative in order to avoid arbitrary generation of band artifacts. In our work the deconvolution was used simply to identify the approximate position of the main component bands. The deconvolved spectra were always compared with the original and with the difference spectra, to ascertain their validity.

For deconvolution, we used a Lorentzian band width of 25 $cm^{-1}$ and a resolution enhancement factor of 1.8, as suggested by Mendelsohn et al. [48]. The deconvolved spectra are shown in Fig. 2. The Amide I band was resolved into three bands located at about 1678 $cm^{-1}$, 1655 $cm^{-1}$, and 1638 $cm^{-1}$. According to customary attribution [48], they correspond to $\beta$ structure (1678 and 1638 $cm^{-1}$) and to $\alpha$ structure (1655 $cm^{-1}$), respectively. Other structures, such as random coil and side chain vibrations, may also contribute to the intensity of the Amide I band. The deconvolved spectra obtained in the absence of DMSO and in the presence of 35% DMSO are similar.

Increasing the concentration of DMSO leads to a series of spectral changes (Figs. 1 and 2A). At 45% DMSO concentration a shoulder appears at about 1630 $cm^{-1}$ (Fig. 1), that is clearly resolved at 1638 $cm^{-1}$ in the deconvolved spectrum (Fig. 2B). The shoulder at 1630–1638 $cm^{-1}$, together with the change in intensity at $\cong$ 1678–1682 $cm^{-1}$, correspond to the formation of new $\beta$ structure. The decrease in the intensity of the band at 1549 $cm^{-1}$ is consistent with greater proton-deuterium exchange.

The difference spectrum (Fig. 2C) also indicates a small decrease in the intensity of the 1650 $cm^{-1}$ band, and the appearance of two small bands at 1670–1680 $cm^{-1}$ and at 1620–1630 $cm^{-1}$. These changes are consistent with a shift of conformational equilibrium from $\alpha$ structures to $\beta$ structures.

Upon raising the concentration of DMSO from 45% to 60% the intensity of the band at 1638 $cm^{-1}$ further increases (Fig. 2B,C), and a new band appears at 1622 $cm^{-1}$. This band is certainly not a deconvolution artifact, since it is well resolved in the original spectrum of SR containing 70% DMSO (Figs. 1 and 2A). The intensity of the 1622 $cm^{-1}$ band is weak or negligible at DMSO concentrations below 60%, but increases sharply between 60% and 70% DMSO, and it is best seen in the spectra of SR containing 70% DMSO (Figs. 1 and 2A,B,C). Concomitantly with the increase of the new band at 1622 $cm^{-1}$ in the presence of 70% DMSO, the Amide I band shifts from 1645 $cm^{-1}$ to about 1639 $cm^{-1}$, with a decrease in its intensity (Fig. 1) and a shoulder develops at 1682 $cm^{-1}$.

Several mechanisms may contribute to these spectral changes.

(1) There may be a shift in the conformational equilibrium of membrane proteins at high concentration of DMSO from the dominant $\alpha$ structure, indicated by the negative $\approx$ 1650 $cm^{-1}$ band in the difference spectrum of Fig. 2C to new $\beta$ structures suggested by the increase in the $\approx$ 1680 and in the $\approx$ 1620–1630 $cm^{-1}$ bands on the difference spectrum (Fig. 2C).

(2) The change in the relative intensity of the $\approx$ 1680 $cm^{-1}$ and the $\approx$ 1650 $cm^{-1}$ bands (Fig. 2C) may also result from a weakening of the hydrogen bonds involving peptide carbonyl groups at high DMSO concentration. DMSO can form hydrogen bonds either with the NH-groups or with water, in competition with the carbonyl groups, as suggested by Khurgin and his colleagues [49].

(3) The 1622 $cm^{-1}$ band is probably not related to $\beta$ structure, since a distinct $\beta$ structure band (1638 $cm^{-1}$) is still seen in the deconvolved spectrum in the presence of 70% DMSO (Fig. 2B). The deconvolved spectra show two resolved bands located, respectively, at 1638 and 1622 $cm^{-1}$, in the 1640–1620 $cm^{-1}$ region usually attributed to $\beta$ structures. Since with increasing concentration the ratio of the intensities of the 1622/1638 $cm^{-1}$ bands increases, we suggest that the 1638 $cm^{-1}$ band corresponds mainly to $\beta$ structure, while the 1622 $cm^{-1}$ band is due in part to side chain (-COOH) vibrations.

To define further the origin of the 1622 $cm^{-1}$ band, the FTIR spectra of bovine serum albumin were analyzed in $D_2O$ medium and in DMSO solutions (Fig. 3). The sharp band at 1622 $cm^{-1}$ together with the shoulder at $\cong$ 1680 $cm^{-1}$, were clearly observed in the spectra of serum albumin in the presence of DMSO, indicating that these features are not unique for membrane proteins. The maximum of the main Amide I peak decreased in intensity and shifted from 1647 $cm^{-1}$ to 1657 $cm^{-1}$ in the presence of DMSO, due in part to the extra intensity at $\cong$ 1687 $cm^{-1}$. The shoulder seen in sarcoplasmic reticulum at $\cong$ 1630–1638 $cm^{-1}$ cannot be clearly identified because of the changes in band positions. After removal of DMSO by dialysis against $D_2O$ medium the 1622 $cm^{-1}$ band completely disappeared, the intensity of the 1657 $cm^{-1}$ band was largely recovered, and shifted to its normal position as seen in $D_2O$ medium.

The 1622 $cm^{-1}$ band was also observed in amino acid mixtures containing aspartate and glutamate, and it is largely attributable to the -COOH groups of these two amino acids. The p$K$ of acetic acid changes from

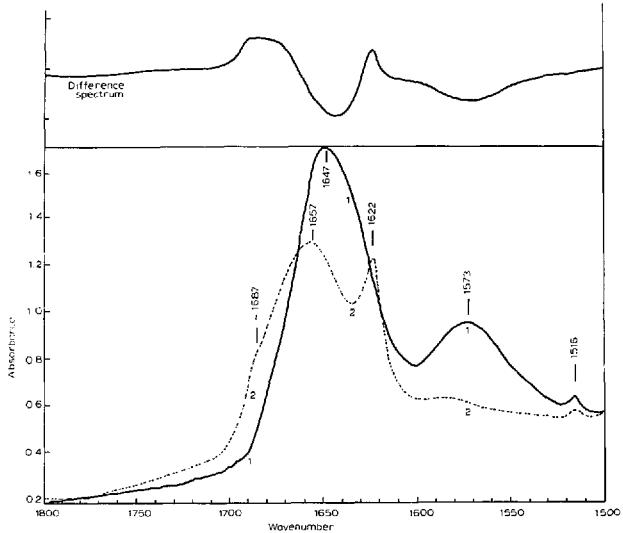


Fig. 3. The effect of DMSO on the FTIR spectrum of bovine serum albumin. Serum albumin (100 mg/ml) was dissolved in $D_2O$ medium (line 1, ——) or in DMSO (line 2, ·····) and analyzed under conditions described in Methods.

4.75 in $H_2O$ to 12.3 in DMSO [50]. Therefore the decrease in the intensity at 1573 $cm^{-1}$ and the increased intensity at 1622 $cm^{-1}$ in the presence of high concentration of DMSO may originate from a decrease in the ionization of the carboxyl groups of aspartate and glutamate. The sharp contour of the 1622 $cm^{-1}$ band is consistent with side chain vibration.

The serum albumin underwent nearly complete proton-deuterium exchange prior to the analysis, as indicated by the low absorption at 1550 $cm^{-1}$, hence addition of DMSO causes only a relatively slight change in this region.

The peak at 1575 $cm^{-1}$ in the sarcoplasmic reticulum may also be due to side chain ($-COO^-$) vibrations (Fig. 1). The decrease in the intensity of this band in the presence of DMSO may be explained, at least in part, by a shift of these vibrations toward higher frequencies. The change at 1575 $cm^{-1}$ in the case of sarcoplasmic reticulum is somewhat overshadowed by the large change in the intensity of the Amide II peak at $\cong$ 1550 $cm^{-1}$, due to the increased proton-deuterium exchange induced by DMSO.

*The effects of dimethyl sulfoxide on ATPase activity*

It is of interest to correlate the changes in FTIR spectra shown in Figs. 1 and 2 with the inhibition of ATPase activity by DMSO (Table I). For ATPase activity measurements the samples were diluted 4000-fold to final DMSO concentrations of less than 0.02%. At these dilutions the effect of DMSO on the ATPase activity is negligible, therefore the ATPase activity measurements reveal the irreversible effects of DMSO on the sarcoplasmic reticulum. Up to $\cong$ 35% DMSO concentration the ATPase activity was largely preserved (Table I), and apart from increased H-D exchange, there were no significant changes in the FTIR spectra (Figs. 1 and 2). Progressive irreversible inhibition of ATPase activity occurred above 40% DMSO concentration that became complete at 70% DMSO, in accordance with the observations of The and Hasselbach [30]. The inhibition

TABLE I

*ATPase activity of sarcoplasmic reticulum after exposure to various DMSO concentrations*

Microsomes (20 mg protein/ml) were incubated at 2°C in a $D_2O$ medium containing 0.1 M KCl, 10 mM imidzole (pD 7.4), 1 mM $MgCl_2$ with DMSO at the indicated concentration. After incubation, the samples were diluted 4000-fold with $H_2O$ medium and the rate of hydrolysis was measured by coupled enzyme assay as described in Methods. The DMSO concentration at final dilution was less than 0.02%. The loss of ATPase activity corresponds to the irreversible inactivation of the enzyme by DMSO.

| [DMSO]during preincubation, % (v/v) | ATPase activity ($\mu$mol $P_i \cdot mg^{-1} \cdot min^{-1}$) |
|---|---|
| 0 | 2.40 ± 0.08 |
| 30 | 2.15 ± 0.20 |
| 35 | 2.18 ± 0.42 |
| 40 | 1.88 ± 0.19 |
| 45 | 1.33 ± 0.18 |
| 50 | 0.89 ± 0.30 |
| 55 | 0.52 ± 0.08 |
| 60 | 0.60 ± 0.09 |
| 70 | 0.00 |

of ATPase activity was accompanied by the increased intensity of the band at about 1630 $cm^{-1}$ (Figs. 1 and 2), suggesting the formation of new $\beta$ or random coil structure, that may be related to the loss of ATPase activity. Above 60% DMSO concentration the ATPase is nearly completely inhibited (Table I). Therefore the appearance of the 1622 $cm^{-1}$ band is not correlated with changes in ATPase activity, but signals the next step of denaturation. After removal of DMSO the 1622 $cm^{-1}$ band disappears, but the ATPase activity remains fully inhibited.

*The influence of DMSO on the pressure sensitivity of $Ca^{2+}$-ATPase*

The FTIR spectrum of sarcoplasmic reticulum in a $D_2O$ medium containing 1 mM EGTA is shown in Fig. 4A before and after pressure treatment at 1.5 kbar for 1 h. After exposure to 1.5 kbar pressure, the Amide I band of SR develops a shoulder at about 1630 $cm^{-1}$, that is clearly resolved both in the difference spectrum (Fig. 4A) and in the deconvolved spectrum (Fig. 5) with a maximum at 1636 $cm^{-1}$. These changes were accompanied by a large irreversible loss of ATPase activity (Table II).

When the pressure treatment was carried out in the presence of 35% DMSO under otherwise identical conditions, the pressure-induced loss of ATPase activity was completely prevented (Table II), and no significant pressure-induced change could be seen in the FTIR spectra of SR at 1630–1638 $cm^{-1}$ (Fig. 4B and Fig. 5).

The protection against the pressure-induced changes in ATPase activity and structure afforded by 35% DMSO was similar to that observed in $D_2O$ media containing 1 mM EGTA + 5 mM vanadate (Table II), and greater than that seen in the presence of 2–200 mM $Ca^{2+}$ (Table II) or polyhydroxy compounds such as sucrose, glycerol or ethylene glycol [45]. Addition of 35% DMSO to systems containing 2–200 mM $Ca^{2+}$ provided further protection against the pressure-induced changes of enzymatic activity (Table II).

*Fluorescence changes of fluorescein-5'-isothiocyanate (FITC) covalently attached to the $Ca^{2+}$-ATPase of sarcoplasmic reticulum*

The FITC selectively labels the $Ca^{2+}$-ATPase at lysine 515 near the ATP binding site [17–23]. The fluorescence

TABLE II

*Effect of DMSO on the pressure sensitivity of $Ca^{2+}$-ATPase*

Microsomes (20 mg protein/ml) were incubated at 2°C in a $D_2O$ medium containing 0.1 M KCl, 10 mM imidazole (pD 7.4), 1 mM $MgCl_2$, with the indicated additions. After exposure to 1 bar (control) or to 1.5 kbar pressure for 1 h, the samples were diluted 4000-fold with $H_2O$ medium and the ATPase activities were measured as described in Methods. The sarcoplasmic reticulum vesicles were labeled with FITC after pressure treatment at a protein concentration of 2 mg/ml, as described by Papp et al. [47]. The calcium-induced fluorescence change represents the percent increase in fluorescence intensity upon addition of 70 $\mu$M $Ca^{2+}$ to a sarcoplasmic reticulum suspension (50 $\mu$g protein per ml) in 100 mM KCl, 5 mM $MgCl_2$, 10 mM K-Mops (pH 7.0) and 35 $\mu$M EGTA.

| Additions | ATPase activity ($\mu$mol $P_i \cdot mg^{-1} \cdot min^{-1}$) | | $Ca^{2+}$-induced fluorescence change (%) | |
|---|---|---|---|---|
| | 1 bar | 1.5 kbar | 1 bar | 1.5 kbar |
| 1 mM EGTA | 4.45 | 0.90 | 6.12 | 0.66 |
| 2 mM $Ca^{2+}$ | 5.18 | 2.10 | 5.18 | 1.53 |
| 20 mM $Ca^{2+}$ | 4.51 | 2.09 | 4.80 | 1.32 |
| 200 mM $Ca^{2+}$ | 5.10 | 1.54 | 4.80 | 0.69 |
| 1 mM EGTA + 5 mM vanadate | 4.24 | 4.41 | 4.70 | 3.87 |
| 1 mM EGTA + 35% DMSO | 4.51 ± 0.49 | 4.92 ± 0.10 | 4.18 | 4.90 |
| 2 mM $Ca^{2+}$ + 35% DMSO | 4.63 ± 0.16 | 4.59 ± 0.18 | 5.71 | 5.62 |
| 20 mM $Ca^{2+}$ + 35% DMSO | 4.81 ± 0.23 | 4.43 ± 0.38 | 5.10 | 4.43 |
| 200 mM $Ca^{2+}$ + 35% DMSO | 4.92 ± 0.23 | 4.62 ± 0.19 | 4.90 | 5.54 |
| 1 mM EGTA + 5 mM vanadate + 35% DMSO | 4.42 ± 0.79 | 3.60 ± 0.21 | 4.10 | 4.28 |

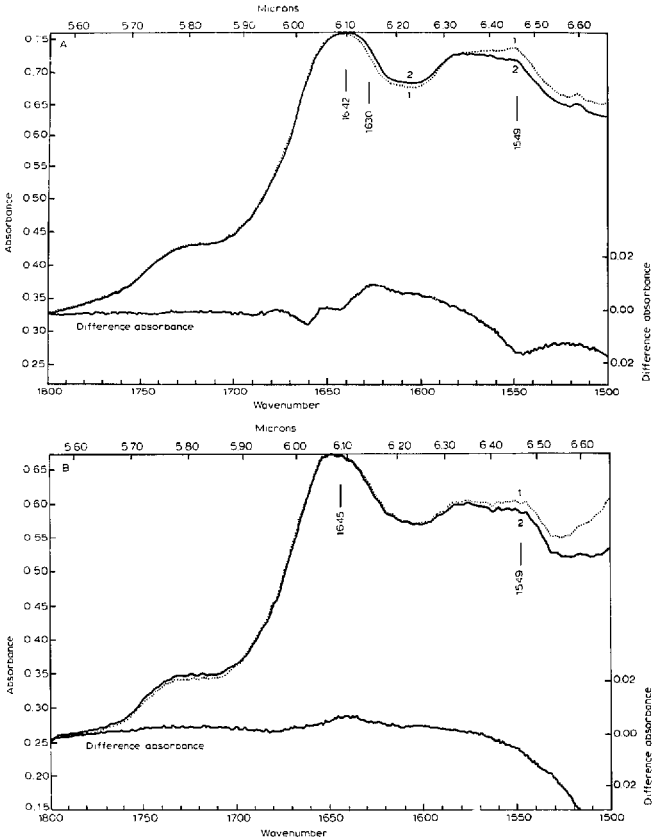


Fig. 4. FTIR spectra of sarcoplasmic reticulum before and after pressure treatment in $D_2O$ medium containing 1 mM EGTA. (A) Top panel, IR spectrum of SR vesicles (15 mg/ml protein) in $D_2O$ medium containing 1 mM EGTA (for composition, see Fig. 1). Control sample kept at 1 bar (line 1, ·····). Sample after pressure treatment at 1.5 kbar for 1 h (line 2, ———). The spectra have been corrected for water vapor. The path length was 50 μm and windows were $BaF_2$. The bottom line corresponds to the difference spectrum. (B) Bottom panel, IR spectrum of SR vesicles (20 mg/ml protein) in $D_2O$ medium containing 1 mM EGTA and 35% DMSO in addition to the components listed above. Control sample kept at 1 bar (line 1, ·····). Sample after exposure to 1.5 kbar pressure for 1 h (line 2, ———). The bottom line corresponds to the difference spectrum.

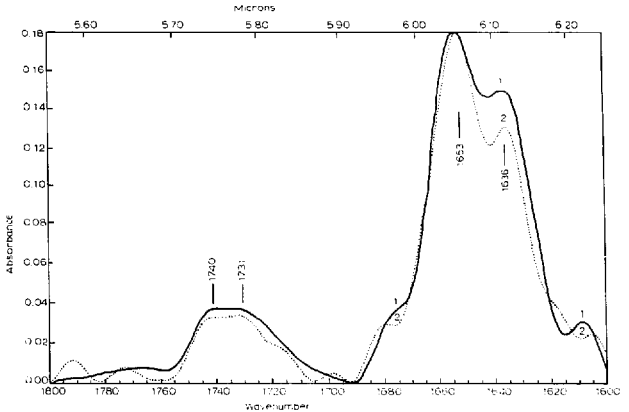


Fig. 5. Deconvoluted spectra of sarcoplasmic reticulum after pressure treatment with or without DMSO. The spectra were obtained after pressure treatment of sarcoplasmic reticulum (1.5 kbar, 1 h) in a $D_2O$ medium containing 1 mM EGTA (line 1, ——) or 1 mM EGTA and 35% DMSO (line 2, ·····). Other medium components are listed in the legend to Fig. 1. A 25 $cm^{-1}$ Lorentzian bandwidth and a resolution factor of 1.8 were used for deconvolution.

intensity of covalently bound FITC decreases upon binding of $Ca^{2+}$ to the high-affinity $Ca^{2+}$ binding site of the $Ca^{2+}$-ATPase and increases upon removal of this $Ca^{2+}$ by EGTA, providing a convenient approach for monitoring the conformational transitions of the enzyme related to enzymatic activity. The $Ca^{2+}$-induced change in the intensity of FITC fluorescence was largely abolished after pressure treatment of sarcoplasmic reticulum at 1.5 kbar for 1 h in media containing 1 mM EGTA or 200 mM $Ca^{2+}$ (Table II). The presence of 2–20 mM $Ca^{2+}$ or 1 mM EGTA + 5 mM vanadate during pressure treatment provided partial protection against the irreversible loss of the fluorescence response to $Ca^{2+}$ and EGTA (Table II). The $Ca^{2+}$-induced fluorescence response was protected when 35% DMSO was included in the medium during pressure treatment (Table II), together with protection of the ATPase against changes in structure (Figs. 4 and 5) and ATPase activity (Table II).

### *The effect of urea on the structure and stability of the $Ca^{2+}$-ATPase*

The effect of urea on the mechanism of $Ca^{2+}$-dependent ATP hydrolysis by sarcoplasmic reticulum is in several respects antagonistic to that of DMSO [32]. Consistent with these observations, urea (0.8 M) in the

TABLE III

*Effect of DMSO and urea on the pressure sensitivity of $Ca^{2+}$-ATPase*

SR vesicles (15–20 mg protien/ml) were suspended at 2°C in $D_2O$ medium containing 0.1 M KCl, 10 mM imidazole (pD 7.4), 1 mM $MgCl_2$ with the indicated additions. After exposure to 1 bar or 1.5 kbar for 1 h at 2°C the samples were diluted 3000- to 4000-fold in $H_2O$ medium and the rate of ATP hydrolysis measured by coupled enzyme assay as described in Methods.

| Additions | ATPase activity ($\mu mol\ P_i \cdot mg^{-1} \cdot min^{-1}$) | |
|---|---|---|
| | 1 bar | 1.5 kbar pressure for 1 h |
| 1 mM EGTA | 3.60 | 0.10 |
| 1 mM EGTA + 0.8 M urea | 1.96 ± 0.06 | 0.00 |
| 1 mM EGTA + 5 mM vanadate | 3.86 | 3.19 |
| 1 mM EGTA + 5 mM vanadate + 0.8 M urea | 3.07 ± 0.43 | 2.41 ± 0.32 |
| 1 mM EGTA + 35% DMSO | 3.60 | 2.84 ± 0.18 |
| 1 mM EGTA + 35% DMSO + 0.8 M urea | 2.95 ± 0.69 | 2.39 ± 0.43 |
| 1 mM EGTA + 5 mM vanadate + 35% DMSO | 2.84 | 2.51 ± 0.13 |
| 1 mM EGTA + 5 mM vanadate + 35% DMSO + 0.8 M urea | 2.84 ± 0.53 | 1.83 ± 0.70 |
| 20 mM $Ca^{2+}$ | 3.80 | 0.70 ± 0.02 |
| 20 mM $Ca^{2+}$ + 0.8 mM urea | 3.11 ± 0.46 | 0.22 ± 0.22 |
| 20 mM $Ca^{2+}$ + 35% DMSO | 3.56 | 2.28 ± 0.36 |

presence of EGTA moderately inhibited the $Ca^{2+}$-ATPase, even at atmospheric pressure, and produced complete loss of ATPase activity at 1.5 kbar pressure within 1 h (Table III). Urea (0.8 M) also decreased the protective effect of 20 mM $Ca^{2+}$, or 1 mM EGTA + 5 mM vanadate, or 1 mM EGTA + 35% DMSO against pressure-induced inactivation (Table III). The loss of ATPase activity during exposure to 1.5 kbar pressure for 1 h in the presence of 1 mM EGTA and 0.8 M urea was accompanied by a decrease in the polarization of FITC fluorescence from 0.35 to 0.16, indicating a 'loosening' of the structure of the ATP binding site, with increase of the rotational freedom of bound FITC. These was less change in the polarization of fluorescence ($p$) after pressure treatment in the presence of 1 mM EGTA, 5 mM vanadate and 0.8 M urea ($p$ = 0.341) or 20 mM $Ca^{2+}$ and 0.8 M urea ($p$ = 0.289), consistent with retention of some ATPase activity (Table III). Therefore vanadate or 20 mM $Ca^{2+}$ counteracted the effects of urea. Due to absorption by urea in the 1700–1600 $cm^{-1}$ range, the effect of urea on the FTIR spectrum of sarcoplasmic reticulum was not analyzed in detail.

## Discussion

Depending on concentration, DMSO produced three types of changes in the FTIR spectra of sarcoplasmic reticulum. Up to about 35% DMSO (v/v), there were no significant changes in the Amide I (1645 $cm^{-1}$) band; the decrease in the intensity of the Amide II (1548 $cm^{-1}$) band was attributed to increasing proton-deuterium exchange and perhaps in part to a change of solvent background (Fig. 1). The increased deuterium exchange in the presence of 35–40% DMSO is consistent with greater accessibility of some hydrophobic sites to water. DMSO, like polyhydroxy compounds [51,52], may also affect directly or indirectly the structure of water at the protein/phospholipid interface, or within the protein. Full ATPase activity could be recovered even after exposure to 40% (v/v) DMSO, in accord with the results of The and Hasselbach [30].

Between 40% and 60% DMSO, a shoulder developed in the Amide I region of the FTIR spectra at about 1630 $cm^{-1}$, that corresponds to a band with a maximum at $\cong$1638 $cm^{-1}$ in the deconvolved spectrum (Fig. 2B). This band is usually attributed to $\beta$ structure [43,48] and its appearance is correlated with the irreversible loss of ATPase activity. Thermal denaturation [53,54], high pH [55] and exposure to high pressure (1.5–2 kbar) at low calcium concentration [56] induce similar changes in the FTIR spectra and in the ATPase activity of sarcoplasmic reticulum. The changes in FTIR spectra caused by DMSO are not unique to membrane proteins, since they have also been observed in globular proteins, including chymotrypsin, trypsin and hemoglobin [49,57].

At very high DMSO concentration (60–70%, v/v), there was a decrease in the intensity of the Amide I band with a shift of its maximum from 1645 $cm^{-1}$ to 1639 $cm^{-1}$ and new bands appeared at 1622 $cm^{-1}$ and 1682 $cm^{-1}$. These changes suggest a shift of conformational equilibrium from the dominant $\alpha$ structures, characterized by the 1650–1655 $cm^{-1}$ band, to the new structures represented by the 1622 $cm^{-1}$ and 1682 $cm^{-1}$ bands (Fig. 2B,C). The abrupt appearance of the 1622 $cm^{-1}$ band at 60–70% DMSO concentration has the characteristics of phase transition due to dehydration either of the lipid phase or of the protein. The 1622 $cm^{-1}$ band is attributed in part to decreased ionization of the aspartyl and glutamyl carboxyl groups of the protein in the presence of DMSO. Upon rehydration of the system by removal of DMSO the 1622 $cm^{-1}$ band disappeared and the intensity of the Amide I band at 1645 $cm^{-1}$ was largely restored, but the shoulder at $\cong$1630 $cm^{-1}$ remained. Although these changes were reversible, the ATPase activity was not restored upon removal of 70% DMSO. The peak of the phospholipid band located at 1728 $cm^{-1}$ in the control sample shifted to about 1735 $cm^{-1}$ in the presence of 70% DMSO. This change is consistent with decreased hydrogen bonding between water and phospholipids. A similar shift in the FTIR spectrum has been observed upon drying of SR vesicles [52].

These observations may have interesting functional implications in the mechanism of $Ca^{2+}$ transport. DMSO facilitates the phosphorylation of the enzyme by inorganic phosphate [31]. It has been suggested that the catalytic site of the $Ca^{2+}$-ATPase is hydrophobic [58] and that organic solvents such as DMSO increase the partition coefficient of $P_i$ from the aqueous phase into the hydrophobic environment, causing an increase in the apparent affinity of the enzyme for inorganic phosphate [32,33]. The suppression of the ionization of carboxyl groups in the vicinity of the active site by DMSO would have a similar effect. The decreased ionization of carboxyl groups in DMSO-water mixtures [50] may explain that the DMSO-induced decrease in the apparent $K_m$ of the enzyme for $P_i$ is greater at alkaline than at acidic pH values; as a result the pH dependence of enzyme phosphorylation by $P_i$ becomes less pronounced in the presence of DMSO, and similar phosphoenzyme levels can be obtained at pH 6 and 7.5 [32].

Two kinds of water molecules are associated with proteins: internal water that fills cavities within the protein, and a peripheral hydration shell that may contain 0.25–0.75 g water per g protein [59]. Based on the analysis of hydration shells of protein crystals, about 42% of the peptide carbonyl oxygens, 44% of the side chain oxygens and nitrogens and $\cong$14% of the peptide

N-H groups are hydrated [60]. The charged groups of aspartic and glutamic acids bind on the average ≅ two water molecules, those of lysine, arginine, glutamine and asparagine one water molecule, and serine, threonine, tyrosine and tryptophan 0.3–0.8 water molecules [60]. Lowering the hydration of lysozyme from 0.9 to ≅ 0.3 g water/g protein inhibits its catalytic activity [61]. Although similar data on the $Ca^{2+}$-ATPase are not available, it is reasonable to assume that the hydrophilic domains of the ATPase representing ≅ 2/3 of its mass would show comparable behavior. Decreased hydration may also play a role in the reversible inhibition of $Ca^{2+}$-ATPase at a DMSO concentration of 30–40% [30], while the partial reactions involving the formation of phosphoprotein from $P_i$ are facilitated [31–33]. Although no detectable change in secondary structure accompanied these effects, the enhanced proton-deuterium exchange in the presence of DMSO suggests increased exposure of peptide NH groups to solvent.

Measurements of the transfer free energies of anions and cations between water and water-organic solvent mixtures indicate that dimethyl sulfoxide weakens, while urea stabilizes interactions between anions and water [32,62]. The opposite is true for cations. Such effects were invoked to explain the increased solubility of inorganic phosphate in organic solvents (35% benzene, 65% isobutyl alcohol), the increased affinity of $P_i$ for the $Ca^{2+}$-ATPase in the presence of DMSO and the opposing effects in the presence of urea [32]. Anionic side chain groups of the protein may be similarly affected, producing greater dehydration of the protein by DMSO-water mixtures than would be expected from the decrease in water activity alone.

The appearance of a shoulder at 1630 $cm^{-1}$ at 40–60% DMSO concentration is satisfactorily explained by the formation of new $\beta$ or random coil structure, although changes in side chain vibrations may also contribute. The shift of the Amide I band to higher frequency at high DMSO concentration may be due to the formation of weaker hydrogen bonds of the peptide CO group and stronger hydrogen bonds by the peptide NH groups in the presence of DMSO, compared with hydrated structures [49]. A similar explanation may be considered for the decrease in the intensity at 1645 $cm^{-1}$ and the increase at 1682 $cm^{-1}$ in sarcoplasmic reticulum vesicles exposed to DMSO (Fig. 2C).

The use of DMSO concentrations as high as 70% is justified by the observation of a rapid recovery of nerve conduction after blockage in vivo by treatment with 50–75% DMSO [63], that was accompanied by reversible changes in the hydration of the myelin structure [64].

Sarcoplasmic reticulum vesicles exposed to 1.5 kbar pressure for 1 h at 2°C in the presence of EGTA lose most of their ATPase activity, the fluorescence response of covalently bound FITC to $Ca^{2+}$ is largely abolished, and the appearance of a shoulder at 1630 $cm^{-1}$ in the FTIR spectra indicates the formation of new $\beta$ structure or random coil. All these structural changes are prevented when the pressure treatment is carried out in the presence of 35% DMSO, indicating that DMSO induces a more stable conformation of the $Ca^{2+}$-ATPase. Similar stabilization against high pressure was observed earlier in the presence of vanadate, $Ca^{2+}$, or polyhydroxy compounds [45,56].

The structural changes during compression of proteins by high pressure are related primarily to packing defects at the interfaces between oligomeric assemblies of proteins, between proteins and the membrane phospholipids, or within individual protein molecules [65]. The pressure denaturation of ribonuclease A [66] and of metmyoglobin [67] could not be satisfactorily explained by exposure of non-polar groups to water, leading to the conclusion that pressure denaturation is not dominated by hydrophobic effects.

Based on these observations, the protection of $Ca^{2+}$-ATPase against pressure-induced inactivation by vanadate was attributed to a more compact structure of the enzyme in the $E_2$-V conformation stabilized by vanadate than in the conformational equilbrium that exists in the presence of EGTA [45,56]. Improved atomic packing at the interfaces between ATPase molecules may also be invoked to explain the stabilization of $Ca^{2+}$-ATPase by $Ca^{2+}$ or polyhydroxy compounds [45,56,68] and by DMSO (this report).

DMSO may alter the structure of water at the protein/water interface or within the protein, thereby reducing the access of water to hydrophobic groups during pressure treatment. The stabilization of $Ca^{2+}$-ATPase by DMSO was weakened in the presence of 0.8 M urea. The key to these opposing effects may be that DMSO is only a hydrogen bond acceptor, while urea can serve either as acceptor or donor in H-bond formation. Urea weakens hydrophobic interactions between nonpolar groups and water, and increases the affinity of solvent for amide and peptide groups [69]. As a result, DMSO and urea are expected to stabilize distinct types of dipole–dipole or dipole–induced dipole interactions.

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