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The effect of high pressure on the conformation, interactions and activity of the Ca^{2+}-ATPase of sarcoplasmic reticulum

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High pressure (100–150 MPa) increases the intensity and polarization of fluorescence of FITC-labeled Ca^{2+}-ATPase in a medium containing 0.1 mM Ca^{2+}, suggesting a reversible pressure-induced transition from the E_1 into an E_2-like state with dissociation of ATPase oligomers. Under similar conditions but using unlabeled sarcoplasmic reticulum vesicles, high pressure caused the reversible release of Ca^{2+} from the high-affinity Ca^{2+} sites of Ca^{2+}-ATPase, as indicated by changes in the fluorescence of the Ca^{2+} indicator, Fluo-3; this was accompanied by reversible inhibition of the Ca^{2+}-stimulated ATPase activity measured in a coupled enzyme system of pyruvate kinase and lactate dehydrogenase, and by redistribution of Prodan in the lipid phase of the membrane, as shown by marked changes in its fluorescence emission characteristics. In a Ca^{2+}-free medium where the equilibrium favors the E_2 conformation of Ca^{2+}-ATPase the fluorescence intensity of FITC-ATPase was not affected or only slightly reduced by high pressure. The enhancement of TNP-AMP fluorescence by 100 mM inorganic phosphate in the presence of EGTA and 20% dimethylsulfoxide was essentially unaffected by 150 MPa pressure at pH 6.0 and was only slightly reduced at pH 8.0. As the enhancement of TNP-AMP fluorescence by P_i is associated with the Mg^{2+}-dependent phosphorylation of the enzyme and the formation of $Mg \cdot E_2$-P intermediate, it appears that the reactions of Ca^{2+}-ATPase associated with the E_2 state are relatively insensitive to high pressure.

These observations suggest that high pressure stabilizes the enzyme in an E_2-like state characterized by low reactivity with ATP and Ca^{2+} and high reactivity with P_i. The transition from the E_1 to the E_2-like state involves a decrease in the effective volume of Ca^{2+}-ATPase.

Introduction

The Ca^{2+}-stimulated hydrolysis of ATP by the Ca^{2+}-ATPase of sarcoplasmic reticulum is inhibited by high hydrostatic pressure [1–6]. Similar inhibition was also observed with *p*-nitrophenyl phosphate [7,8] and dinitrophenyl phosphate [9,10] as substrates.

After brief exposure to pressures up to about 150 MPa the inhibition of ATPase activity is largely reversible. Above 150 MPa pressure irreversible inhibition of Ca^{2+}-ATPase occurs particularly in Ca^{2+}-free media at low temperatures [3,5]. The loss of ATPase activity under these conditions was accompanied by changes in the tryptic digestion profiles, SH group reactivity and infrared spectra of the Ca^{2+}-ATPase and by inhibition of the conformational responses of the FITC-labeled enzyme after the addition of Ca^{2+} or EGTA + vanadate [5,11,12]. Ca^{2+} (2–20 mM), ATP (5 mM) and EGTA + vanadate protected the Ca^{2+}-ATPase against the irreversible pressure-induced inactivation, implying greater stability at high pressure in the liganded Ca_2E_1 and E_2V conformations than in the conformational equilibrium that exists in EGTA-containing media in the absence of vanadate [5]. Protection was also observed in solutions containing su-

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Abbreviations: $C_{12}E_8$, octaethylene glycol dodecyl ether; SR, sarcoplasmic reticulum; FITC, fluorescein 5′-isothiocyanate; FITC-SR, FITC-labeled sarcoplasmic reticulum; Fluo-3, fluorescein derivative of the EGTA: calcium indicator; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; NADH, β-nicotinamide adenine dinucleotide, reduced form; Mops, 3-(*N*-morpholino)propanesulfonic acid; Prodan, 6-propionyl-2-(dimethylamino)naphthalene; Tris, tris(hydroxymethyl)aminomethane; P_i, inorganic orthophosphate; TNP-AMP, 2′(3′)-trinitrophenyladenosine 5′-monophosphate; DMSO, dimethylsulfoxide; LDH, lactate dehydrogenase; PK, pyruvate phosphokinase; PEP, phospho*enol*pyruvate.

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crose, glycerol, ethylene glycol or KCl (1 M each), suggesting that water density modifying groups impart stability on the enzyme under pressure [3,5].

The activation volume calculated from the pressure dependence of the reversible inhibition of *p*-nitrophenyl phosphate and dinitrophenyl phosphate hydrolysis at saturating substrate concentration was pressure dependent, increasing from ≅ 20 ml/mol at 0.1 MPa to ≅ 80 ml/mol at 100 MPa [8]. In the same pressure range the apparent binding volume for Mg-*p*-nitrophenyl phosphate and for Mg-dinitrophenyl phosphate decreased from 20 ml/mol to ≅ 0 ml/mol, while the binding volume of Ca^{2+} increased from 35–67 ml/mol to 155 ml/mol [8,9]. The pressure dependence of the activation volume at saturating substrate and Ca^{2+} concentrations indicates that the rate-limiting step of the reaction is different at low and at high pressure [13]. The pressure dependence of the rate limiting step will alter the equilibria between the elementary steps of the reaction cycle; this is likely to be expressed in pressure-dependent structural changes of the system caused by the accumulation and depletion of structurally distinct reaction intermediates of the Ca^{2+}-ATPase.

The reversible inhibition of ATPase activity at high pressure may be caused by:

(a) reversible changes in the secondary and tertiary structure of the Ca^{2+}-ATPase monomer that affect its intramolecular dynamics, solvation, substrate binding and catalytic properties [13–17];

(b) reversible dissociation of ATPase oligomers with indirect effects on their stability and activity [1,6,18];

(c) changes in the structure of the lipid phase [2,19–22].

The relative importance of the various mechanisms in the volume changes associated with ATP hydrolysis is analyzed in this report using covalently attached fluorescein 5'-isothiocyanate as a probe of the conformation and interactions of the Ca^{2+}-ATPase, Fluo-3 as an indicator of the Ca^{2+} binding to the Ca^{2+}-ATPase, and Prodan as an indicator of lipid phase structure.

The results suggest pressure-induced changes of the structure of the lipid phase and of the Ca^{2+}-ATPase that lowers the Ca^{2+} affinity of the enzyme with inhibition of the ATP-dependent reactions associated with the E_1 conformation, without major effect on the phosphorylation of the enzyme by P_i in the E_2 state.

Experimental procedures

Materials

Fluorescein 5'-isothiocyanate, Fluo-3, TNP-AMP and Prodan were obtained from Molecular Probes, Eugene, OR 97402. The detergent $C_{12}E_8$ was the product of Calbiochem Diagnostics Corp., La Jolla, CA 92037. KCl and Na_3VO_4 were purchased from Fisher Scientific, Fair Lawn, NJ 07410; the lactate dehydrogenase (rabbit muscle), pyruvate kinase (rabbit muscle), phospho*enol*pyruvate, imidazole, Tris-maleate, Tris, NADH, Mops were from Sigma Chemical Company, St. Louis, MO 63178.

Methods

Preparation of sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum (SR) vesicles were isolated from white skeletal muscles of female rabbits according to Nakamura et al. [23]. The microsome preparations were stored until use at −70°C in a medium of 300 mM sucrose and 10 mM Tris-maleate (pH 7.0). For protein determination the method of Lowry et al. [24] was used employing bovine serum albumin as standard.

Fluorescence measurements

Fluorescence measurements were carried out using a SLM 4800 spectrophotofluorometer equipped with two emission monochromators. The fluorometer was interfaced to a computer to collect and store data for evaluation. A rhodamine B quantum counter was used in the reference beam to obtain corrected excitation spectra and to compensate for variations in the excitation light intensity.

The high pressure fluorescence cell (HPSC-3K) was purchased from SLM Instruments (Urbana, IL) and the Bourdon-type pressure gauge from Ashcroft, Dresser Ind. (Stratford, CT). The hand operated high pressure generator, the high pressure valves, connections and tubes were the products of High Pressure Equipment Co. (Erie, PA). The hydraulic fluid was dehydrated ethanol. The pressure cell was thermostated using a circulating water bath (Model RH-6, Lauda Div., Brinkmann Instrument Co., Westbury, NY) and the temperature was checked by a thermometer placed close to the sample in the hole of the pressure vessel. Before measurements 10–20 min were allowed for temperature equilibration.

Polarization of fluorescence was determined in T configuration, using Glan-Thompson prism polarizers. The measured polarization values were corrected for the scrambling coefficients of the windows, determined according to Palladini and Weber [25], using laser grade fluorescein in glycerol. For each pressure at least 20 sets of polarization data were collected with standard deviations of ≅ 0.001. For intensity measurements and for analysis of fluorescence spectra the polarizers were removed to avoid polarization artifacts.

The standard buffer for fluorescence measurements was 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-Mops (pH 7.0) with further additions and changes as noted in the legends.

To detect irreversible changes caused by high pressure several pressure cycles were used, both for intensity and polarization measurements. The pressure was increased from 0.1 MPa in 20–50 MPa increments and at each pressure data were collected for several minutes to establish a steady pattern of optical response. After the maximal pressure was reached the pressure was returned in the same decrements to 0.1 MPa.

ATPase activity

The Ca^{2+}-stimulated ATPase activity was determined using coupled enzyme assay in a medium of 100 mM KCl, 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.45 mM $CaCl_2$, 0.5 mM EGTA, 5 mM ATP, 0.42 mM PEP, 7.5 U/ml PK, 18 U/ml LDH, 1 μM A23187 and 200 μM NADH at a protein concentration of 5 or 10 μg/ml. Measurements were carried out at 23°C, as described earlier [5].

To determine the enzymatic activity at different pressures, the fluorescence of NADH was followed in the pressure cell at 460 nm using light of 340 or 360 nm for excitation. The changes in fluorescence intensity were measured at each pressure for 40–180 s to determine the slope and the average fluorescence intensity. The fluorescence changes were influenced by inner filter effects that decreased in magnitude with the decrease in the concentration of NADH as the reaction progressed. To apply correction for the inner filter effects the enzymatic activity was also determined by absorption measurements at 340 nm in samples of identical composition at 0.1 MPa over a reaction time of 12–15 min. Since throughout this time the reaction rate remained constant, the fluorescence data were corrected for the inner filter effect, using the intensity-slope function measured at 0.1 MPa. The corrected data were expressed as μmoles of ATP hydrolyzed per mg protein per min, using an absorption coefficient of $6.22 \cdot 10^3$ M^{-1} cm^{-1} for NADH.

All components of the coupled enzyme system used for ATPase activity measurements were present in sufficient concentration to assure a linear relationship between ATP hydrolysis and NADH oxidation throughout the measurements. The activity of lactate dehydrogenase is not influenced measurably by pressure in the range of 0.1–200 MPa [26,27]. Although the activity of pyruvate kinase is moderately pressure sensitive [14], the enzyme was applied in sufficient excess to render the coupled enzyme assay essentially independent of pressure up to $\cong$ 200 MPa [6,28]. The Tris-HCl buffer was chosen because its pK is relatively insensitive to pressure [13,29] and near pH 7.5 there is only a modest pH dependence of ATPase activity.

Calculation of activation volume

Activation volume of the. Ca^{2+}-ATPase was calculated from data obtained at saturating Ca^{2+} and Mg-ATP concentrations according to the formula:

$$\Delta V^{\ddagger} = \frac{R \cdot T}{P} \cdot \ln \frac{V_P}{V_0}$$

where T is the absolute temperature, R universal gas constant, V_0 the maximum velocity of Ca^{2+}-stimulated ATP hydrolysis at atmospheric pressure, and V_P the ATPase activity at pressure P.

The apparent activation volume of the fluorescence response of the FITC-SR was calculated by the same equation, except that V_P and V_0 were replaced by the fluorescence intensities at atmospheric pressure (ϕ_0), and at pressure P (ϕ_P).

Measurement of the free Ca^{2+} concentration

For measurement of free Ca^{2+} concentration Fluo-3 (1 μM) was used as Ca^{2+} indicator [30,31]. The Fluo-3 fluorescence was excited at 495 nm and the emission was measured at 525 nm. The media usually contained 0.1 M KCl, 5 mM $MgCl_2$, 10 mM Tris-Mops (pH 7.0) with Ca^{2+} and EGTA at concentrations described in the legends. At 0.1 MPa pressure the dissociation constant of Fluo-3 for Ca^{2+} is $\cong$ 396 nM [31]. Since the dissociation constant is pressure-dependent, the relationship of Fluo-3 fluorescence to Ca^{2+} concentration was calibrated for each pressure.

Calculation of free calcium concentration

Ionized Ca^{2+} concentrations (pCa) were calculated using a program kindly provided by A. Fabiato [32,33]. The following stability constants were used: Ca^{2+}·EGTA: $2.48 \cdot 10^7$; Mg · EGTA: $1.32 \cdot 10^2$; Ca-ATP $6.93 \cdot 10^3$; Mg-ATP: $1.61 \cdot 10^4$; K-ATP: 5.99; Na-ATP: 7.49. Total Ca^{2+} concentration was determined by atomic absorption spectrometry in a Perkin-Elmer model 3030 spectrometer.

Results

The pressure dependence of the rate of ATP hydrolysis

The rate of Ca^{2+}-stimulated ATP hydrolysis by native sarcoplasmic reticulum vesicles (Fig. 1A; circles) declined with increasing pressure from $\cong$ 6.3 μmol mg^{-1} min^{-1} at 0.1 MPa to $\cong$ 0.8 μmol mg^{-1} min^{-1} at 100 MPa. Similar inhibition of ATPase activity was observed at high pressure in vesicles solubilized with $C_{12}E_8$ (Fig. 1A; squares). Although the equilibrium constant of the Mg-ATP complex is moderately pressure-dependent [8,34], the concentration of Mg-ATP ($\cong$ 5 mM) was far above the K_m of the Ca^{2+}-ATPase for ATP both at the active site ($\cong$ 10 μM) and at the regulatory site ($\cong$ 0.5 mM), assuring saturation of the

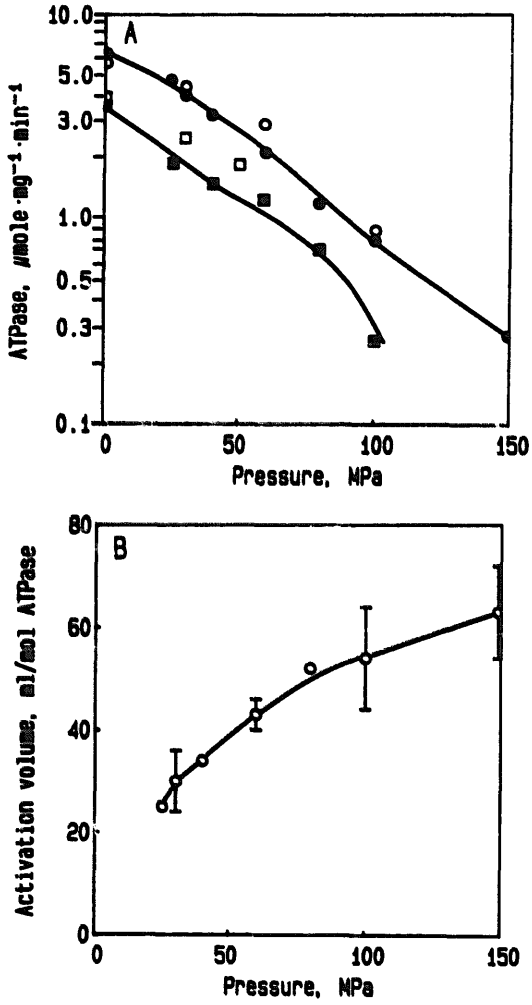

Fig. 1. Pressure dependence of enzymatic activity and activation volume of the Ca^{2+}-ATPase. (A) ATPase activity was determined by the coupled enzyme assay in the absence (●, ○) and in the presence of 2 mg $C_{12}E_8$ per mg SR protein (■, □) as described in Materials and Methods. The filled symbols indicate data obtained with increasing pressure and open symbols data obtained with decreasing pressure. The data points correspond to the mean of two (■, □) or 3–9 (●, ○) independent determinations. The free Ca^{2+} concentration was 1–10 μM, the temperature 23°C and the protein concentration 5 μg/ml. (B) Activation volume of Ca^{2+}-stimulated ATP hydrolysis in the absence of $C_{12}E_8$ (○), based on the data shown under (A) with increasing pressure. The bars indicate standard errors of the mean for data sets that contained more than five independent measurements.

substrate binding site(s) throughout the pressure range employed. Therefore the changes recorded in Fig. 1 represent genuine pressure-induced inhibition of the Ca^{2+}-stimulated ATPase activity. The inhibition of ATP hydrolysis during brief exposure to 100–150 MPa pressure was reversible, both in native vesicles and in solubilized preparations, and after lowering the pressure from 100 MPa to 0.1 MPa the ATPase activity was fully recovered (Fig. 1A). The presence of the Ca^{2+}-ionophore A23187 assured that pressure-induced changes in the Ca^{2+} permeability of the sarcoplasmic reticulum membrane did not contribute to the observed changes in ATPase activity.

There was a nonlinear relationship between the logarithm of ATPase activity and pressure, both in the vesicular and in the solubilized systems (Fig. 1A), indicating that the activation volume of ATP hydrolysis is pressure-dependent. In native sarcoplasmic reticulum preparations the activation volume of ATP hydrolysis increased with pressure from 24 ml/mol at 25 MPa to 53.2 ml/mol at 100 MPa (Fig. 1B). The pressure dependence of the activation volume indicates that the rate-limiting step of the reaction changes with pressure. In sarcoplasmic reticulum preparations solubilized in $C_{12}E_8$ (2 mg/mg protein) the pressure dependence of the activation volume increased steeply from 30 to 95 ml/mol between 60 and 100 MPa (not shown), consistent with the sharp decrease in ATPase activity at the higher pressures (Fig. 1A). These differences between native and solubilized sarcoplasmic reticulum in the pressure dependence of the activation volume suggest that in addition to the volume changes that accompany the reaction of Ca^{2+} and Mg-ATP with the enzyme, some changes in protein-protein and lipid–protein interactions may also contribute to the pressure dependence of the activation volume.

The mechanism of the effect of pressure on the hydrolysis of ATP

The inhibition of ATP hydrolysis at high pressure is likely to be due to a combination of kinetic and equilibrium effects of pressure, that changes the conformation of the Ca^{2+}-ATPase and affects its interaction with Ca^{2+}, ATP and inorganic phosphate [35]. Such a possibility is supported by the reversible pressure-induced dissociation of ATPase oligomers [6] and by the altered susceptibility of the enzyme to trypsin at ≅ 100 MPa pressure that accompanies the inhibition of ATP hydrolysis [10]. Pressure-induced changes in the structure of the lipid phase could also produce such effects [2]. To determine the relative contribution of the various mechanisms to the pressure-induced inhibition of ATPase activity we measured the effect of pressure: (a) on the Ca^{2+}-dependence of ATP hydrolysis; (b) on the conformational equilibrium of the Ca^{2+}-ATPase; and (c) on the structure of the lipid phase of the sarcoplasmic reticulum.

The effect of Ca^{2+} concentration on the inhibition of ATP hydrolysis by pressure

The phosphorylation of the Ca^{2+}-ATPase by ATP requires the saturation of the high-affinity Ca^{2+} binding sites. Therefore the pressure-induced inhibition of ATPase activity may be due to a decrease in the Ca^{2+} affinity of the Ca^{2+}-ATPase at high pressure. This was tested by measuring the pressure dependence of ATP-

ase activity at Ca^{2+} concentrations ranging between 23 nM and 1.25 mM (Figs. 2 and 3).

Optimal ATPase activity required a free Ca^{2+} concentration of $\cong$ 2–3 μM (Fig. 2). Increasing the pressure from 0.1 MPa to 100 MPa caused a progressive decrease in the rate of ATP hydrolysis without changing significantly the Ca^{2+} concentration required for half maximal activation ($\cong$ 0.25 μM). The half-maximal inhibition of ATPase activity was obtained at $\cong$ 40 MPa pressure at all Ca^{2+} concentrations that we tested (Fig. 3). These observations do not support a competitive relationship between calcium and pressure in the regulation of ATP hydrolysis; rather it appears that increasing pressure reversibly decreases the concentration of the Ca^{2+}-ATPase species that is capable of reaction with Ca^{2+} and ATP, without changing the Ca^{2+} affinity of the reactive enzyme form. A plausible interpretation of this effect is that with increasing pressure the conformational equilibrium of the Ca^{2+}-ATPase shifts from the Ca_2E_1 form that is readily phosphorylated by ATP, in favor of the E_2 form of low Ca^{2+} affinity that cannot react with ATP. If this explanation is valid the enzyme should be converted at 100 MPa pressure nearly completely into the E_2 form, in spite of the presence of Ca^{2+}. Since only the Ca_2E_1 species reacts with ATP and translocates Ca^{2+}, the depletion of the E_1 enzyme form will explain the inhibition of ATPase activity at high pressure without change in the K_m(Ca) of ATP hydrolysis.

Alternatively, high pressure may slow the rate of a critical reaction step with or without changes in the $E_1 \rightleftharpoons E_2$ equilibrium. The choice between these and other alternatives will be analyzed in the Discussion.

The inhibition of ATPase activity at Ca^{2+} concentrations in excess of $\cong 10^{-5}$ M (Fig. 2) is usually attributed to inhibition of Ca^{2+} release from the $E_2P \cdot Ca_2$ complex on the luminal side of the sarcoplasmic reticulum, with stabilization of the $E_2P \cdot Ca_2$ enzyme form [35]. This interpretation may require some revision [36]. From inspection of Fig. 2 it appears that with increasing pressure the Ca^{2+} concentration required for half-maximal inhibition of ATPase activity shifts from 80 μM at 0.1 MPa to $\cong$ 10 μM at 100 MPa. These observations would imply that the stability of the $E_2P \cdot Ca_2$ intermediate increases with pressure, that may also contribute to the pressure-induced inhibition of ATPase activity. The mechanism of this effect will be the subject of future study.

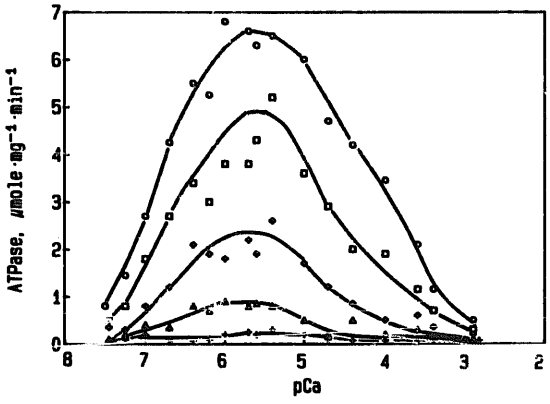

Fig. 2. The dependence of ATPase activity on Ca^{2+} concentration at various pressures. The ATPase activities were determined using the coupled enzyme assay in the high pressure fluorescence cell as described in Methods. The ionized Ca^{2+} concentrations were adjusted by EGTA-Ca^{2+} buffers, and the free Ca^{2+} concentrations were calculated using the program of Fabiato [33]. The measurements were carried out at 23°C at 0.1 MPa (○), 30 MPa (□) 60 MPa (◇), 100 MPa (△) and 150 MPa (+) pressures.

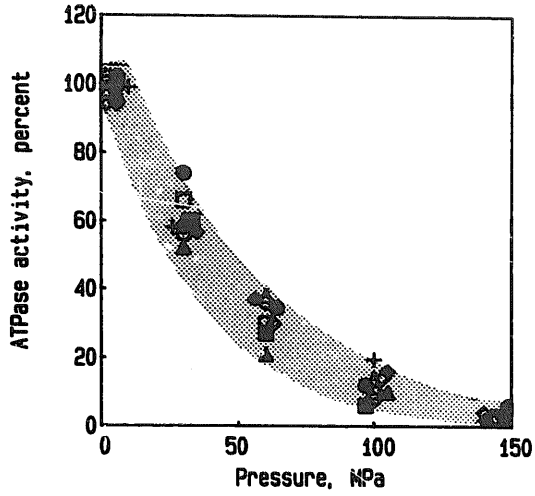

Fig. 3. Pressure dependence of ATPase activity at various Ca^{2+} concentrations. The ATPase activities were determined at increasing pressures using the coupled enzyme assay as described in the legend to Fig. 2, at the following free calcium concentrations: 20–40 nM (○), 90–200 nM (□), 0.4–0.6 μM (△), 1–2 μM (◇), 2.4–4 μM (●), 10–20 μM (■), 40–100 μM (▲), 260–380 μM (◆) and 1.25 mM (+). Each symbol represents 2–4 independent measurements. The data are expressed as percentage of the ATPase activity determined at atmospheric pressure. The normalized data show similar pressure dependence of ATPase activity at all Ca^{2+} concentrations, indicating that the decrease in Ca^{2+}-stimulated ATPase activity is not associated with a detectable shift in the Ca^{2+} affinity of the reactive enzyme form.

The pressure dependence of the conformational equilibrium of the Ca^{2+}-ATPase

The effect of pressure on the conformational equilibrium of Ca^{2+}-ATPase was investigated using vesicular and detergent solubilized sarcoplasmic reticulum preparations in which the Ca^{2+}-ATPase was covalently labeled with FITC at lysine 515. At atmospheric pressure the FITC-labeled preparations respond with a decrease in fluorescence intensity to the addition of 10–100 μM Ca^{2+}, that stabilizes the Ca^{2+}-ATPase in the Ca_2E_1 state (Fig. 4A), and with an increase in fluorescence intensity after the addition of EGTA and vanadate (Fig. 4B), that stabilizes the Ca^{2+}-ATPase in

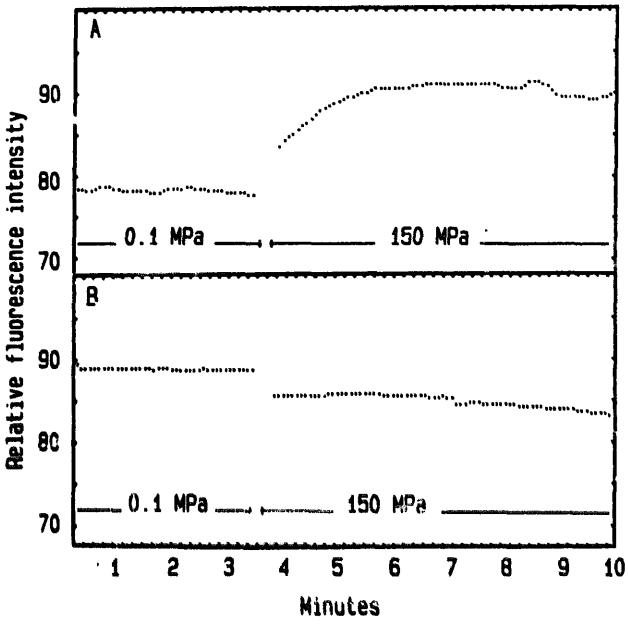

Fig. 4. Effect of pressure on the intensity of fluorescence of FITC-SR. Sarcoplasmic reticulum vesicles were labeled with FITC as described earlier [41], using 5 nmoles FITC/mg SR protein. Labeled vesicles were suspended in a medium of 100 mM KCl, 5 mM $MgCl_2$, 20 mM K^+-Mops (pH 7.0) to 50 μg/ml protein concentration. The E_1 conformation of the enzyme (A) was stabilized by addition of 0.1 mM $CaCl_2$, while the E_2V state (B) was stabilized by addition of 0.1 mM EGTA and 0.5 mM vanadate. Fluorescence of FITC-SR was excited at 470 nm (4 nm slit width) and the emission intensity was monitored at 530 nm (4 nm slit width) at 0.1 MPa; pressure was raised to 150 MPa. Temperature: 20°C.

the E_2V state [37–40]. The difference in fluorescence intensity between the two states is $\cong$ 10%.

The fluorescence intensity of the FITC-labeled Ca^{2+}-ATPase suspended in a Ca^{2+} medium (Ca_2E_1 state) was enhanced by about 10% after increasing the pressure from 0.1 MPa to 150 MPa (Fig. 4A). The halftime of the transition to the new equilibrium was about 1 min, at 20–23°C, and the fluorescence intensity remained stable at that level for at least 5 min. The pressure-induced increase in the fluorescence of FITC-Ca^{2+}-ATPase cannot be attributed to a change in the pH of the solution, since under the same conditions the fluorescence intensity of free fluorescein increased only slightly (Fig. 5A).

In solutions of 0.1 mM EGTA and 0.5 mM sodium vanadate, i.e. under conditions that stabilize the E_2V state of the Ca^{2+}-ATPase, application of 150 MPa pressure caused only a slight decrease in the fluorescence intensity of FITC-labeled Ca^{2+}-ATPase (Fig. 4B).

These observations are consistent with the suggestion that under the influence of high pressure the conformational equilibrium of Ca^{2+} -ATPase shifts from the Ca_2E_1 toward the E_2 state. The $\cong$ 10% increase in fluorescence intensity at 150 MPa shown in Fig. 4A is comparable in magnitude to that produced by the addition of EGTA and vanadate to the FITC-ATPase preequilibrated with Ca^{2+} at 0.1 MPa, when the enzyme is nearly completely converted from the Ca_2E_1 into the E_2V form. Nevertheless, based on these data alone, the possibility remains that the fluorescence emission characteristics of the FITC-ATPase may be modified at high pressure by deformation of the active site without change in the overall conformation of the enzyme.

The relationship of the pressure-induced change in the fluorescence of FITC-Ca^{2+}-ATPase to the change in ATPase activity is further analyzed in Fig. 5. Step-

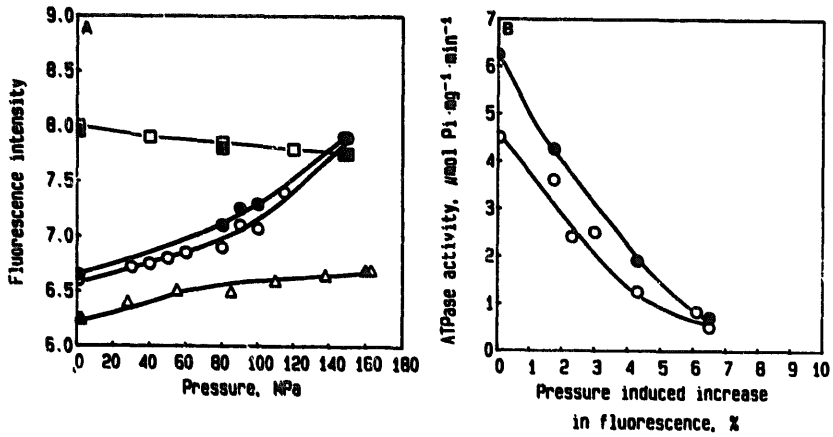

Fig. 5. Pressure dependence of the fluorescence of FITC-labeled sarcoplasmic reticulum. (Panel A) Sarcoplasmic reticulum vesicles were labeled with FITC as described earlier [41] using 5 nmoles of FITC/mg SR protein. The labeled vesicles were suspended in a medium of 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.0) to a final protein concentration of 50 μg/ml. The enzyme was stabilized in the E_1 state (○, ●) by addition of 0.1 mM $CaCl_2$ or in the E_2V state (□, ■) by 0.1 mM EGTA + 0.5 mM Na_3VO_4. The fluorescence of FITC-SR was excited at 470 nm and the emission was monitored at 530 nm using 4 nm slit width in both cases. The same medium containing 0.5 μM fluorescein and no sarcoplasmic reticulum was used as control (△, ▲). Temperature was maintained at 20°C. Open symbols are data obtained at increasing pressure and the closed symbols during release of pressure. (Panel B) The relationship of the ATPase activity of the unlabeled enzyme to the pressure-induced increase in the fluorescence of FITC-SR at pressures ranging between 0.1 and 100 MPa.

wise 20 MPa increments of pressure produced a non-linear increase in the fluorescence intensity of FITC-labeled Ca^{2+}-ATPase in the presence of 0.1 mM Ca^{2+} (Fig. 5A; circles), while there was only a slight decrease in the fluorescence intensity in the E_2V state stabilized by EGTA and vanadate (Fig. 5A; squares). The small increase in the fluorescence of fluorescein with increasing pressure is consistent with volume compression of the sample and with pressure-induced ionization of the dye (see below).

The pressure-induced increase in the fluorescence intensity of FITC-Ca^{2+}-ATPase in the presence of 0.1 mM Ca^{2+} is roughly proportional to the decrease in the ATPase activity of the native enzyme over the pressure range of 0.1–100 MPa (Fig. 5B). The activation volume of the pressure induced conformational transition calculated from the change in FITC fluorescence is also similar to the activation volume of ATP hydrolysis (not shown). This observation is again consistent with the proposition that the inhibition of ATPase activity at high pressure may be caused by a shift in the $E_1 \rightleftharpoons E_2$ equilibrium in favor of the E_2 form, causing the depletion of the E_1 species of the enzyme that reacts with Ca and ATP.

Pressure-induced changes in intrinsic tryptophan fluorescence

The intrinsic tryptophan fluorescence of sarcoplasmic reticulum increases by 3–4% upon saturation of the high affinity Ca^{2+} binding site of the Ca^{2+}-ATPase [40,42,43]. Removal of Ca^{2+} by EGTA causes a corresponding decrease in tryptophan fluorescence. It is not established whether these changes in tryptophan fluorescence represent local changes in the structure of the Ca^{2+}-ATPase associated with the binding of Ca^{2+} or a more general change in the conformation of the enzyme related to the transition between the E_2 and Ca_2E_1 states.

Raising the pressure to 100 MPa had no significant effect on the intrinsic tryptophan fluorescence of the Ca^{2+}-ATPase in sarcoplasmic reticulum vesicles, either in the Ca_2E_1 or in the E_2V states (Fig. 6A, top two lines), although at 100 MPa pressure the Ca^{2+}-stimulated ATP hydrolysis was nearly completely inhibited (Figs. 1 and 2). After increasing the pressure to 150 MPa the fluorescence intensity decreased by 10–15% in both states (Fig. 6B, top two lines); the rate of transition was slower in the presence of EGTA and vanadate. Releasing the pressure to 0.1 MPa restored the fluorescence intensity to the starting level in the vanadate-containing sample (Fig. 6B, dotted line), while in the presence of 0.1 mM Ca^{2+} the reversal of fluorescence was only partial (Fig. 6B, solid line). The irreversible component of the fluorescence change may be related to the time-dependent, pressure-induced inactivation of Ca^{2+}-ATPase at 150 MPa in the presence of

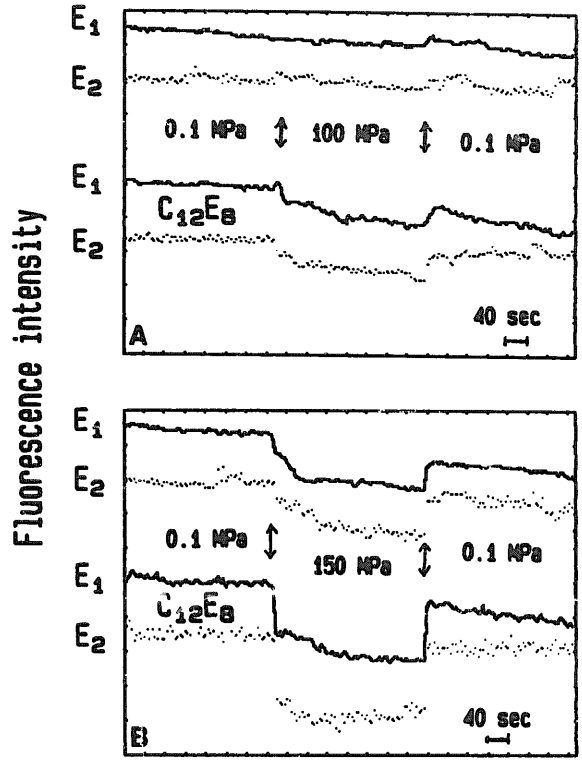

Fig. 6. Effect of pressure on the intrinsic tryptophan fluorescence of sarcoplasmic reticulum. Sarcoplasmic reticulum vesicles were suspended in 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.0) to a final protein concentration of 0.1 mg/ml. The medium also contained either 0.1 mM $CaCl_2$ to stabilize the E_1 conformation (solid lines), or 0.1 mM EGTA + 0.5 mM Na_3VO_4 to stabilize the E_2V conformation of the enzyme (dotted lines). The fluorescence emission was monitored at 335 nm (slit width 8 nm) using exciting light of 295 nm (slit width 2 nm). Temperature: 20°C. The fluorescence intensity was successively recorded at 0.1 MPa, 100 MPa (panel A), 150 MPa (panel B) and again at 0.1 MPa for 5 min at each stage. In both panels the two upper traces represent the vesicular sarcoplasmic reticulum in the E_1 and E_2V states, respectively. The two lower traces were obtained on solubilized sarcoplasmic reticulum preparations containing 2 mg $C_{12}E_8$/mg protein in the Ca_2E_1 (solid line) and in the E_2V states (dotted line). Ordinate divisions correspond to 10% change of the initial fluorescence; the divisions on the abscissa equal 40 s. ATPase activities before pressure treatment ranged between 2.5 and 4.5 μmol mg^{-1} min^{-1}. There was only moderate decrease in ATPase activity in all samples after exposure to 100 MPa. At 150 MPa the ATPase activity remained protected only in the E_2V state, but was irreversibly lost in the Ca_2E_1 state.

0.1 mM Ca^{2+} (Table I). Vanadate afforded complete protection against the irreversible inactivation at 100–150 MPa pressure (Table I), in agreement with earlier observations [5,11,12].

Solubilization of the sarcoplasmic reticulum in $C_{12}E_8$ (2 mg/mg protein) enhanced the pressure-induced decrease in fluorescence intensity, both at 100 MPa (Fig. 6A, bottom two lines) and at 150 MPa (Fig. 6B, bottom two lines). In preparations containing 0.1 mM Ca^{2+} the intensity of tryptophan fluorescence progressively decreased under high pressure; after return to 0.1 MPa there was only a partial recovery of tryptophan fluorescence while the decreasing trend continued (Fig.

TABLE I

Irreversible effects of pressure treatment on ATPase activity

For details on experimental conditions, see Methods.

Addition	$C_{12}E_8$ (2 mg/mg protein)	ATPase activity (μmol mg^{-1} min^{-1})		
		starting material (0.1 MPa)	after treatment at	
			100 MPa	150 MPa
0.1 mM Ca	–	3.3–3.6	2.7	0.2
0.1 mM Ca	+	2.6	1.0	0.0
0.1 mM EGTA and 0.5 mM Na_3VO_4	–	4.2–4.5	3.8	4.2
0.1 mM EGTA and 0.5 mM Na_3VO_4	+	4.3	4.0	–
0.1 mM EGTA and 0.5 mM Na_3VO_4	+	3.2	–	1.7–2.5

6A, B). By contrast, in the presence of EGTA and vanadate the decrease in fluorescence intensity at high pressure was completely reversed after return to 0.1 MPa and there was no significant loss of fluorescence during continued incubation for several min (Fig. 6A, B).

Matching these observations in solubilized ATPase preparations containing 0.1 mM Ca^{2+} there was a substantial irreversible loss of ATPase activity, both at 100 and 150 MPa pressures (Table I); EGTA and vanadate provided complete protection at 100 MPa and significant protection at 150 MPa against irreversible inactivation of Ca^{2+}-ATPase, even in the presence of $C_{12}E_8$ (Table I).

In conclusion, the pressure-induced decrease in the intensity of tryptophan fluorescence occurs only at pressures much higher (150 MPa) than that required for nearly complete reversible inhibition of Ca^{2+}-stimulated ATP hydrolysis in native sarcoplasmic reticulum vesicles. At 150 MPa pressure the fluorescence change does not show any clear relationship to the conformational state of the enzyme prior to pressurization.

Pressure induced changes in the Ca^{2+} affinity of the Ca^{2+}-ATPase

In the E_1 state the Ca^{2+}-ATPase cooperatively interacts with 2 Ca^{2+} ions at the high-affinity Ca^{2+} transport sites with $K_{Ca} = 10^{-7}$ M; in the E_2 state only two low affinity Ca^{2+} binding sites are available, with $K_{Ca} = \cong 10^{-3}$ M (Refs. 35 and 44; see, however, Ref. 36). -The decrease in the Ca^{2+} affinity and the change in the polarity of the Ca^{2+} binding sites after phosphorylation of the enzyme by ATP are key elements of the

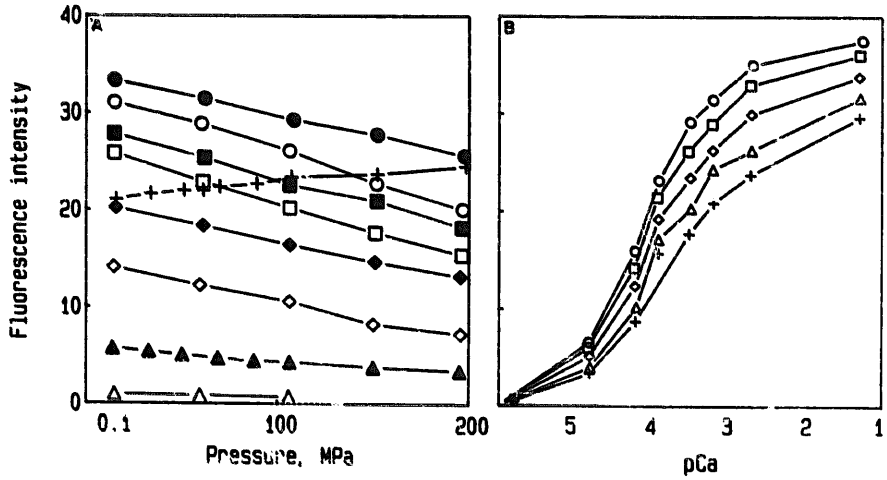

Fig. 7. Pressure dependence of fluorescein and Fluo-3 fluorescence at different Ca^{2+} concentrations. (Panel A) The intensity of fluorescence of Fluo-3 (1 μM) was measured as a function of pressure in a medium of 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-Mops (pH 7.0), after addition of calcium or EGTA at the following concentrations: △, 1 mM EGTA; ▲, no addition ($\cong$ 15 μM Ca^{2+}); ◇, 0.05 mM Ca^{2+}; ◆, 0.1 mM Ca^{2+}; □, 0.3 mM Ca^{2+}; ■, 0.6 mM Ca^{2+}; ○, 2 mM Ca^{2+}; ●, 50 mM Ca^{2+}. For comparison, pressure cycles were also carried out in the same medium using instead of Fluo-3, 1 μM fluorescein (+) as a chemically related fluorescence probe that does not interact with Ca^{2+}. Fluorescence was excited at 495 nm (slit width, 4 nm) and the emission intensity was measured at 525 nm (slit width, 4 nm) at 20°C. (Panel B) The data shown under (A) were replotted as the function of Ca^{2+} concentration at the following pressures (MPa): ○, 0.1; □, 50; ◇, 100; △, 150; +, 200.

coupling between ATP hydrolysis and Ca^{2+} transport [35,44].

If under high pressure the Ca^{2+}-ATPase is forced from the E_1 to the E_2 state, its Ca^{2+} affinity should decrease by several orders of magnitude. This possibility was tested using Fluo-3 as a fluorescent indicator of Ca^{2+} concentration. As a preliminary to these measurements the pressure dependence of the fluorescence of the Ca^{2+}-Fluo-3 complex was analyzed at Ca^{2+} concentrations ranging between > 0.01 μM and 50 mM (Figs. 7A, B). Over the whole range of Ca^{2+} concentrations the stability of the Ca^{2+}-Fluo-3 complex was pressure dependent, as indicated by an $\cong$ 10–16% decrease in fluorescence intensity with 100 MPa change in pressure (Fig. 7A). The decrease in fluorescence intensity at high pressure is largely due to dissociation of the Ca^{2+}-Fluo-3 complex. Half maximal increase in fluorescence intensity in media containing 0.1 M KCl, 5 mM $MgCl_2$, 10 mM Tris-Mops (pH 7.0) and 1 μM Fluo-3 was obtained at $\cong$ 89 μM Ca^{2+} concentration at atmospheric pressure and at $\cong$ 400 μM Ca^{2+} at 200 MPa pressure (Fig. 7B). Under the same conditions the fluorescence intensity of free fluorescein increased by $\cong$ 6% per 100 MPa increase in pressure (Fig. 7A). Some of this increase is explained by volume decrease due to compression (2–3.6% per 100 MPa), but some small change may arise from pressure-induced decrease in the pK of fluorescein. The pressure dependence of the fluorescence of free fluorescein was similar in the E_1 or E_2 media in the presence or absence of sarcoplasmic reticulum.

For measurement of Ca^{2+} binding to the Ca^{2+}-ATPase, sarcoplasmic reticulum vesicles were depleted of much of their contaminating calcium and were suspended in media containing 0.1 M KCl, 5 mM $MgCl_2$, 10 mM Tris-Mops (pH 7.0) and 1 μM Fluo-3, either without vanadate (Fig. 8, solid line; Ca_2E_1 state) or with 1 mM vanadate (Fig. 8, dotted line; E_2V state). The total Ca^{2+} concentration was 10 μM in both samples. The difference in Ca^{2+}-Fluo-3 fluorescence between the vanadate-free and vanadate-containing samples (Fig. 8) reflects the decrease in free Ca^{2+} concentration caused by Ca^{2+} binding to the high affinity Ca^{2+} sites of the Ca^{2+}-ATPase in the E_1 state. The rapid decrease in fluorescence intensity after each pressure step indicates pressure-induced dissociation of Ca^{2+}-Fluo-3 complex that is followed by readjustment to a new equilibrium. With increasing pressure the difference between the vanadate-containing and vanadate-free samples was reduced to about one half at 100 MPa and became near zero at 200 MPa. Since up to 100–150 MPa there is little irreversible inhibition of ATPase activity, these observations are consistent with the pressure-induced conversion of the Ca^{2+}-ATPase from the $Ca_2 \cdot E_1$ into the E_2 state of low Ca^{2+} affinity. The alternative explanation that the E_2V

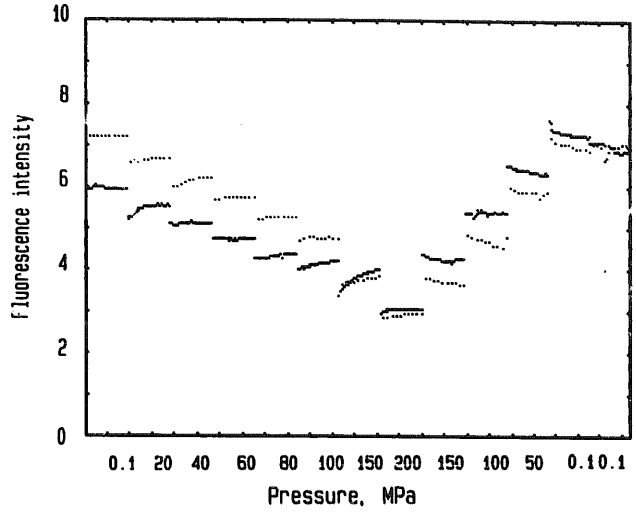

Fig. 8. Effect of pressure on the fluorescence of Fluo-3 in the presence and absence of vanadate. Fluorescence intensity was excited at 495 nm and the emission intensities were measured at 525 nm in a medium of 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-Mops (pH 7.0), and 1 μM Fluo-3 without added Ca^{2+}. The total concentration of Ca^{2+} originating from the various reagents was about 10 μM. Protein concentration was 1 mg/ml. Vesicles were washed prior to the experiment in the presence of 10 μM A23187 and 0.1 mM EGTA to lower the Ca^{2+} contamination, followed by washing in standard medium. Solid lines correspond to the intensity data obtained in the absence of vanadate (E_1 state); the dotted line represents to data obtained in the presence of 1 mM Na_3VO_4 (E_2-like state). The pressure was increased stepwise to 150 MPa and then decreased stepwise to 0.1 MPa. The fluorescence intensity was measured for 150 seconds at each step; then pressure was set to the next value and the intensity of fluorescence determined again. Pressure values were: 0.1, 20, 40, 60, 80, 100, 150, 200, 150, 100, 50, 0.1 MPa and after 15 minutes of incubation, 0.1 MPa again.

form of the Ca^{2+}-ATPase would acquire Ca^{2+} binding ability at high pressure is not likely. At 200 MPa pressure the vanadate-free Ca^{2+}-ATPase is inactivated at the low Ca^{2+} concentration ($\cong$ 10 μM) used in these experiments [5], and as a result it is unable to bind the released Ca^{2+} during return to atmospheric pressure (Fig. 8, solid line). Vanadate prevents the irreversible inactivation of the enzyme [5] and in the vanadate-containing samples some Ca^{2+} may have been bound during return from 200 MPa to 0.1 MPa pressure (Fig. 8, dotted line) explaining the crossing over of the two curves during the return phase.

The pressure dependence of the binding of Ca^{2+} to the Ca^{2+}-ATPase in the E_1 and E_2 states was also compared at 20 and 50 μM total Ca^{2+} concentrations, in the pressure range of 0.1–100 MPa, where no irreversible inactivation of the Ca^{2+}-ATPase occurs (Fig. 9). The rapid decrease of fluorescence intensity after each pressure step again indicates the dissociation of Ca^{2+}-Fluo-3 complex that is fully reversed during return to atmospheric pressure. The difference in the amplitude of the fluorescence signal between vanadate-containing and vanadate-free samples at 20 μM

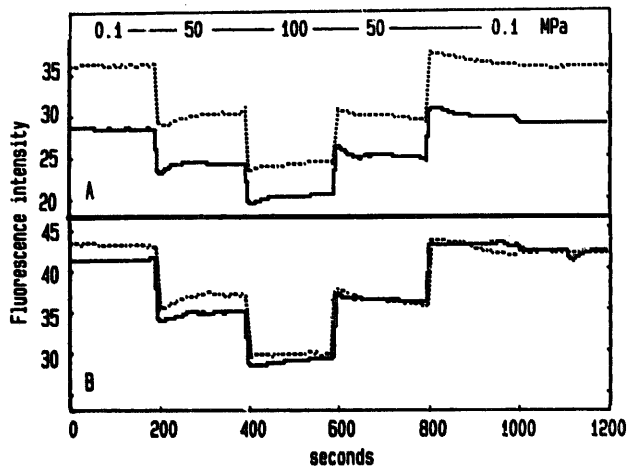

Fig. 9. Pressure dependence of Fluo-3 fluorescence at different Ca^{2+} concentrations in the presence of sarcoplasmic reticulum. The fluorescence intensity of Fluo-3 was measured at 525 nm using 495 nm light for excitation in a medium of 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-Mops (pH 7.0) and 1 mg SR protein/ml. The sarcoplasmic reticulum vesicles were washed before the experiment in the same medium containing 0.1 mM EGTA and 10 μM A23187 to reduce Ca^{2+} contamination and to permeabilize the membrane; this was followed by a second wash in the same medium but without EGTA or A23187, prior to the addition to the test system. Pressure was increased stepwise from 0.1 MPa to 50 and to 100 MPa, then decreased in the same steps to 0.1 MPa. Measurements were made at total Ca^{2+} concentrations of $\cong$ 20 μM (panel A), and 40 μM (panel B). Solid lines: no vanadate; dashed lines: data obtained under identical conditions with 1 mM Na_3VO_4 added.

total Ca^{2+} concentration was 27% of the total fluorescence intensity at 0.1 MPa and decreased to 16% at 100 MPa (Fig. 9A and Fig. 10). This decrease is consistent with release of Ca^{2+} from the Ca_2E_1 form of the Ca^{2+}-ATPase. After releasing the pressure the fluorescence intensity returned near to its starting level in both samples, indicating the reversibility of the pressure effect. At 50 μM total Ca^{2+} concentration the difference between vanadate-free and vanadate-containing samples became smaller as the bound Ca^{2+} became a smaller fraction of the total Ca^{2+} concentration (Fig. 9B and Fig. 10).

Although the pressure-induced decrease in the Ca^{2+} binding capacity of Ca^{2+}-ATPase is consistent with a shift in equilibrium in favor of the E_2 state characterized by low affinity for Ca^{2+}, local effects of pressure on the structure of the Ca^{2+} binding site could also explain the observations with or without a change in conformational equilibrium.

Pressure dependence of the reaction of Ca^{2+}-ATPase with inorganic orthophosphate

If the inhibition of Ca^{2+}-stimulated ATP hydrolysis at high pressure is indeed due to the stabilization of the E_2 conformer of the Ca^{2+}-ATPase, then the phosphorylation of the enzyme by inorganic orthophosphate in the E_2 state should either be unaffected or promoted by pressure, depending on the Ca^{2+} concentration of the medium.

This problem was approached (Fig. 11) utilizing the observation [45] that the fluorescence intensity of TNP-AMP bound to the active site of the Ca^{2+}-ATPase increases after phosphorylation of the enzyme by inorganic phosphate. At 30 mM P_i concentration, the enhancement of TNP-AMP fluorescence was 3.5–4 fold at pH 6.0–8.0, compared with control samples that did not contain inorganic phosphate (Fig. 11). At pH 6.0, where the affinity of the enzyme for P_i is highest, the enhancement of TNP-AMP fluorescence changed only moderately with increasing pressure up to 150 MPa (Fig. 11). Increasing the pH to pH 7.0 and 8.0 reduced the affinity of the enzyme for P_i [35] and increased the pressure sensitivity of the fluorescence enhancement (Fig. 11); at pH 8.0 the fluorescence enhancement caused by 30 mM P_i was reduced to $\cong$ 1/3 at 50 MPa and was essentially abolished at 100 MPa (Fig. 11).

These observations indicate that the phosphorylation of the enzyme by P_i, is only moderately sensitive to pressure under conditions where the P_i binding site of the enzyme is saturated. The decrease in the P_i-induced enhancement of TNP-AMP fluorescence at high pressure at pH 7.0–8.0 (Fig. 11) may be caused by inhibition of the binding of P_i or TNP-AMP to the enzyme or by inhibition of the phosphorylation reaction. Some inhibition of TNP-AMP binding at high

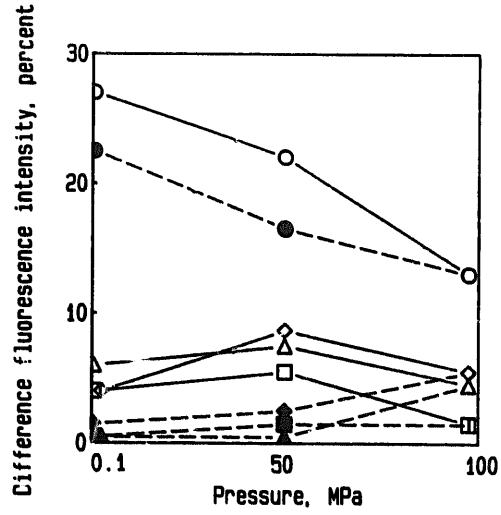

Fig. 10. Difference fluorescence of Fluo-3 at various pressures caused by the binding of vanadate to sarcoplasmic reticulum. The fluorescence of Fluo-3 was measured at 525 nm (4 nm slit width) using light of 495 nm for excitation (4 nm slit width). The measurements were made in a medium of 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-Mops (pH 7.0) in the presence and absence of 1 mM Na_3VO_4 without addition of Ca^{2+} (free Ca^{2+} $\cong$ 10 μM) (○, ●) or with $CaCl_2$ added to final concentrations of 20 μM (□, ■) or 50 μM (△, ▲) or 80 μM (◇, ◆). The difference in fluorescence intensities between vanadate-containing and vanadate-free samples are shown on the ordinate, expressed as a percentage of the total fluorescence. Open and filled symbols represent data obtained during increase and decrease of pressure, respectively.

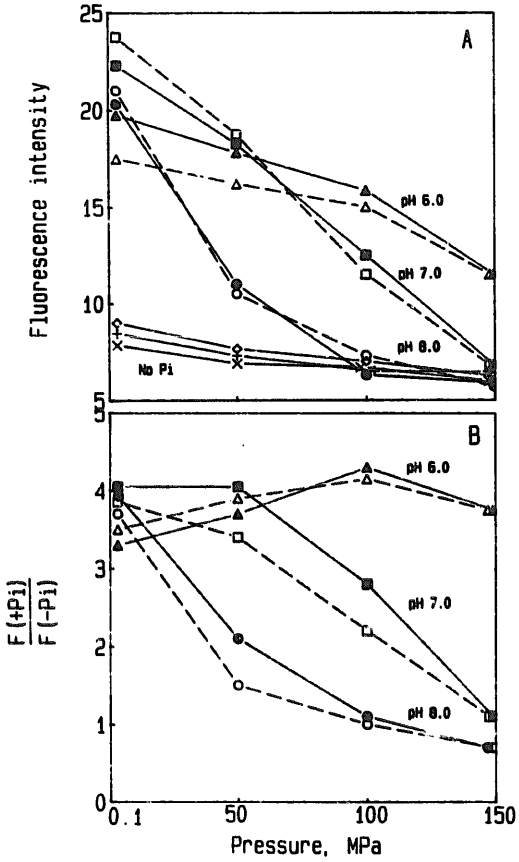

Fig. 11. Pressure dependence of the fluorescence of TNP-AMP bound to sarcoplasmic reticulum in the presence of DMSO. Leaky SR vesicles were prepared according to [46]. Vesicles were suspended in a medium of 50 mM Tris-maleate, 10 mM $MgCl_2$, 1 mM EGTA+20% DMSO to a final concentration of 0.1 mg SR protein/ml. Fluorescence intensities were measured at pressures of 0.1, 50, 100, 150, 100, 50 and 0.1 MPa at 540 nm while excited at 408 nm. TNP-AMP concentration was 1 μM. All measurements were carried out at 20°C in a pressure bomb, as described in Materials and Methods, in the presence of 30 mM phosphate (□, ■, ○, ●, △, ▲), or in the absence of phosphate (◇, +, ×). Filled symbols correspond to data obtained by increasing the pressure, while open symbols by releasing the pressure, at pH 6.0 (▲, △), pH 7.0 (■, □), and pH 8.0 (●, ○). (A) Fluorescence intensity as a function of pressure. (B) Ratio of intensity of fluorescence in the presence of 30 mM phosphate over in the absence of phosphate as a function of pressure.

pressure is indicated by the slight decrease in TNP-AMP fluorescence in the absence of P_i (Fig. 11A).

To test whether the pressure effect can be overcome by increase in the concentration of inorganic phosphate, the experiments shown in Fig. 11 were repeated at 100 mM inorganic phosphate concentration (Fig. 12). Under these conditions the P_i-induced enhancement of TNP-AMP fluorescence was essentially unaffected by 0.1–150 MPa pressure at pH 6.0 and was only slightly reduced at pH 7–8. These observations are consistent with the hypothesis that the E_2 conformation of the enzyme is stable at 150 MPa pressure in Ca^{2+}-free medium in the presence of inorganic phosphate.

The effect of pressure on ATPase–ATPase interactions in native and solubilized sarcoplasmic reticulum

In native sarcoplasmic reticulum membranes the Ca^{2+}-ATPase forms oligomeric assemblies that impart cooperative kinetics on the Ca^{2+}-stimulated ATP hydrolysis and protect the Ca^{2+}-ATPase against denaturation [47]. After labeling the Ca^{2+}-ATPase with FITC and illuminating the membranes with polarized exciting light the emitted fluorescence becomes depolarized due to energy transfer between adjacent fluorescein molecules in oligomers of FITC-labeled Ca^{2+}-ATPase [48]. Consistent with this interpretation the polarization of the emitted fluorescence is larger after solubilization of the membranes with detergents that disrupts the ATPase oligomers and increases the average distance between ATPase molecules beyond the range of efficient energy transfer [48]. Therefore the polarization of fluorescence of covalently bound FITC can be used as an indicator of ATPase–ATPase interactions.

This technique was applied to the analysis of the effect of pressure on the stability of the Ca^{2+}-ATPase oligomers both in native and in detergent solubilized sarcoplasmic reticulum membranes at Ca^{2+} concentrations of 0.01, 2, and 20 mM (Figs. 13A, B and C) and in a Ca^{2+} free media with or without 5 mM vanadate (Figs. 14A and B).

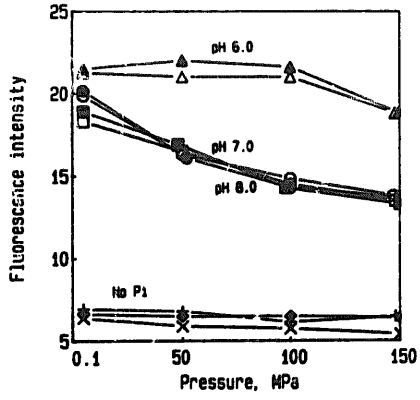

Fig. 12. Pressure dependence of the fluorescence of TNP-AMP in sarcoplasmic reticulum suspensions in the presence of DMSO. Leaky sarcoplasmic reticulum vesicles were prepared according to [46]. The measurements were made at 22°C in a medium of 50 mM Tris-maleate, 10 mM $MgCl_2$, 1 mM EGTA, 20% DMSO and 100 mM inorganic phosphate when indicated, at a final protein concentration of 100 μg/ml. The pH was adjusted to 6.0, 7.0 or 8.0. The fluorescence was excited at 408 nm and the emission intensity was recorded at 540 nm. The pressure was adjusted stepwise to 0.1, 50, 100, 150, 100, 50 and 0.1 MPa. The data are plotted using the following symbols: Bottom lines: no inorganic phosphate at pH 6.0 (◆), pH 7.0 (+) and pH 8.0 (×). Top and middle lines: 100 mM inorganic phosphate at pH 6.0 (△, ▲), pH 7.0 (□, ■) and pH 8.0 (○, ●). Filled symbols represent data obtained during increasing pressure and open symbols during decreasing pressure.

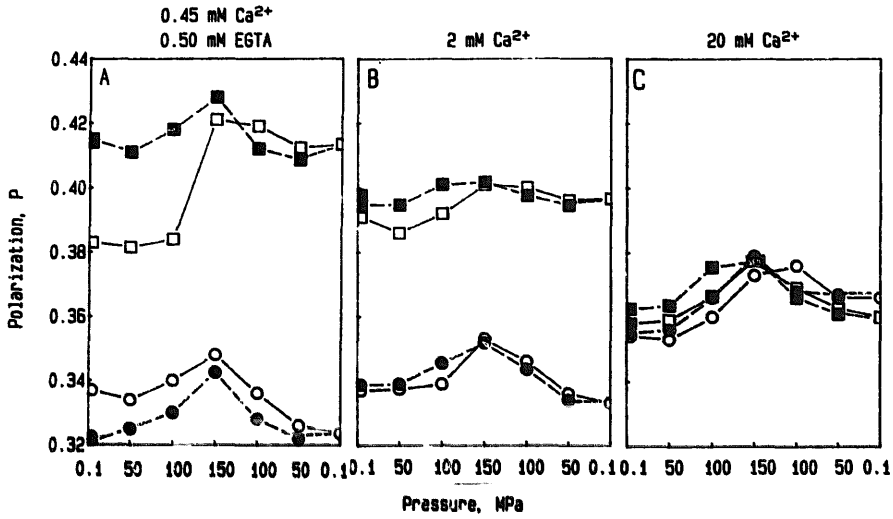

Fig. 13. Effect of pressure on the polarization of fluorescence of FITC-SR in the presence of Ca^{2+}. Sarcoplasmic reticulum vesicles were labeled with FITC as described earlier [41]. The medium contained 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-Mops (pH 7.0) and 50 μg/ml of FITC-SR, with further additions given below. Pressure was set consecutively to 0.1, 50, 100, 150, 100, 50, and 0.1 MPa for two successive pressure cycles, and at each given pressure the polarization of fluorescence was determined at 20°C. The following additions were made: 0.45 mM Ca^{2+} + 0.5 mM EGTA (panel A), 2 mM Ca^{2+} (panel B), 20 mM Ca^{2+} (panel C). Symbols: no detergent (○, ●); 2 mg $C_{12}E_8$/mg protein (□, ■). Open symbols (solid lines) represent the polarization measured in the first pressure cycle; filled symbols (broken lines) are data obtained in the second pressure cycle.

ATPase–ATPase interactions in the presence of Ca^{2+}

Increase in pressure from 0.1 MPa to 150 MPa *reversibly* increased the polarization of FITC fluorescence in native and in solubilized membranes by $\Delta P \cong 0.01$ (Figs. 13A, B and C). This effect was largely independent of Ca^{2+} concentration and since it was observed both in the absence or presence of detergents, it is probably not related to ATPase–ATPase interactions. The reversible increase in polarization at high pressure may be due to restricted mobility of FITC in the compressed protein matrix [49].

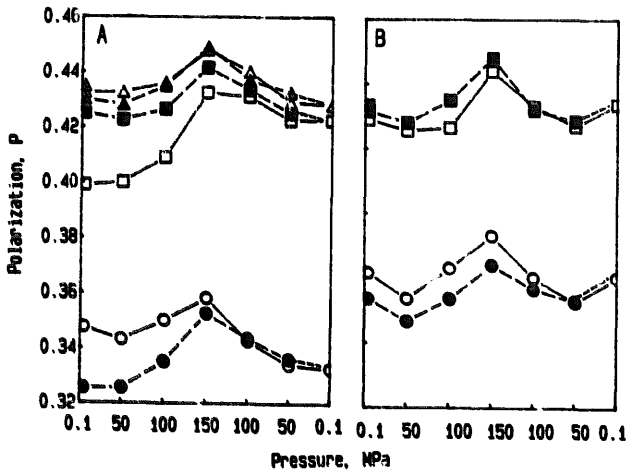

Fig. 14. Effect of pressure on the polarization of FITC fluorescence in the absence of calcium. (A) Sarcoplasmic reticulum vesicles were labeled with FITC as described earlier [41], using 5 nmol FITC/mg SR. The FITC-SR was suspended in a medium of 100 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-Mops (pH 7.0), and 0.5 mM EGTA in the presence of 2 mg $C_{12}E_8$/mg SR (□, ■) or 10 mg $C_{12}E_8$/mg SR (△, ▲). Identical samples without detergent were included as control (○, ●). Protein concentration: 50 μg/ml. The samples were preincubated for 30 min in the presence of detergent at 20°C and the polarization of fluorescence determined at 0.1, 50, 100, 150, 100, 50 and 0.1 MPa pressure (open symbols). The pressure cycle was repeated one more time (filled symbols). Each point represents an average of 20 polarization measurements on any given sample, with the standard error of the mean usually 0.0015 or less. (B) A similar experiment to A except that the medium also contained 5 mM Na_3VO_4, either without $C_{12}E_8$ (○, ●) or with 2 mg $C_{12}E_8$/mg protein (□, ■).

Solubilization of the membranes by $C_{12}E_8$ at moderate Ca^{2+} concentration (0.01–2 mM) caused a large increase in the polarization of fluorescence ($\Delta P \cong 0.03$) that we attribute to the dissociation of ATPase aggregates into ATPase dimers (Figs. 13A and B; open squares, and Ref. 50). In samples containing 10^{-5} M Ca^{2+} (Fig. 13A; open squares), a further increase in fluorescence polarization occurred after application of 150 MPa pressure that brought the level of polarization ($P \cong 0.42$) near the theoretical maximum expected for monomeric ATPase solutions. This change was not reversed after lowering the pressure to 0.1 MPa and was not observed during the next pressure cycle (Fig 13A; filled squares), indicating that the putative ATPase dimers did not reform at atmospheric pressure under the conditions of these experiments.

In samples containing 2 mM Ca^{2+} the secondary pressure-induced increase in polarization was not observed (Fig. 13B; open squares). The level of polarization remained near $P = 0.40$ even at 150 MPa pressure, suggesting that the Ca^{2+}-ATPase dimers are stabilized against pressure-induced disruption by 2 mM Ca^{2+}.

At 20 mM Ca^{2+} concentration addition of $C_{12}E_8$ (2 mg/mg protein) caused no significant increase in fluorescence polarization (Fig. 13C; open squares) com-

pared with detergent-free control samples (Fig. 13C; open circles), either at atmospheric or at 150 MPa pressure, indicating powerful stabilization of ATPase–ATPase interactions by 20 mM Ca^{2+}. This is consistent with our earlier observations that 20 mM Ca^{2+} promotes the formation of crystalline Ca^{2+}-ATPase aggregates in detergent solutions [50,51]. The polarization of FITC fluorescence in detergent-free systems at 0.1 MPa was significantly higher at 20 mM Ca^{2+} ($P \cong 0.36$; Fig. 13C) than at 0.01–2 mM Ca^{2+} concentration ($P \cong 0.34$; Figs. 13A and B), suggesting some Ca^{2+}-dependent change in the structure of the Ca^{2+}-ATPase.

ATPase–ATPase interactions in the presence of EGTA

Solubilization of the sarcoplasmic reticulum membranes by 2 mg $C_{12}E_8$/mg protein in the presence of 0.5 mM EGTA increased the polarization of fluorescence at atmospheric pressure from $\cong 0.35$ to 0.40 (Fig. 14A, open squares). This change is similar to that observed in solutions containing 0.01–2 mM Ca^{2+} (Figs. 13A and B). The secondary dissociation caused by raising the pressure to 150 MPa in the presence of 2 mg $C_{12}E_8$/mg protein was also observed in Ca^{2+}-free medium (Fig. 14A, open squares). Therefore the biphasic dissociation of Ca^{2+}-ATPase oligomers by detergents is not dependent on Ca^{2+} binding to the high affinity Ca^{2+} sites of the Ca^{2+}-ATPase. The small hysteresis of polarization in the native, low Ca^{2+} samples (Figs. 13A and 14A) might be due to partial inactivation of the enzyme [12].

At a higher detergent concentration (10 mg $C_{12}E_8$/mg protein) the dissociation of ATPase oligomers was complete already at atmospheric pressure and increasing the pressure to 150 MPa produced only a small reversible increase in polarization that was independent from the state of association of Ca^{2+}-ATPase (Fig. 14A, triangles).

A more detailed analysis of the effect of $C_{12}E_8$ on the polarization of fluorescence of FITC-ATPase is shown in Fig. 15 at $C_{12}E_8$/protein weight ratios of 0, 0.25, 0.5, 1, 2, 5, 20 and 100. In the absence of detergents the polarization of fluorescence at 0.1 MPa ranged between 0.32 at 100 μg protein/ml to 0.35 at 1 μg protein/ml (Fig. 15, open symbols). This difference may reflect light scattering depolarization, that is more pronounced at the higher protein concentrations [52]. After one pressure cycle the polarization decreased below starting level, while the second pressure cycle produced largely reversible changes in fluorescence polarization (Fig. 15, open symbols). A significant increase in fluorescence polarization was seen at atmospheric pressure already at a detergent: protein ratio as low as 0.25, that caused only partial solubilization of the membrane. Under these conditions the small increase in polarization at 150 MPa pressure was nearly fully reversible (Fig. 15; +). At higher detergent/protein ratios (1–2 mg $C_{12}E_8$/mg protein) the polarization of fluorescence increased to $\cong 0.38$ at atmospheric pressure (Fig. 15; ◆, ●), the further increase to $\cong 0.43$ at 150 MPa was only partially reversed after the first cycle. Above 5 mg $C_{12}E_8$/mg protein full dissociation of ATP oligomers occurred already at 0.1 MPa pressure, and the pressure-induced changes in polarization became fully reversible (Fig. 15; ▲, ■).

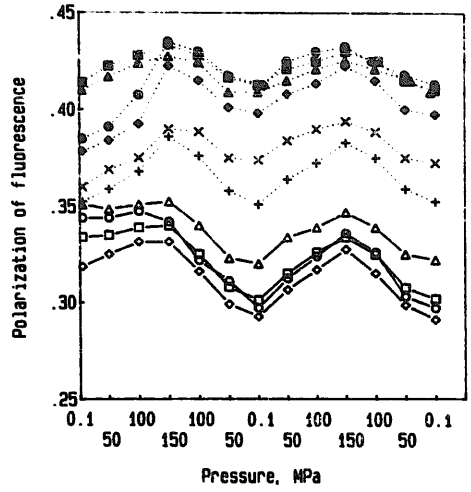

Fig. 15. The polarization of FITC-SR fluorescence at various detergent protein ratios. Sarcoplasmic reticulum vesicles were labeled with FITC as described in [41]. The polarization of fluorescence was measured at 525 nm using 480 nm light for excitation. The medium contained 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.0), 0.45 mM $CaCl_2$, 0.5 mM EGTA and FITC-SR in concentrations of 1 μg/ml (△, ▲), 5 μg/ml (□, ■), 50 μg/ml (○, ●), 100 μg/ml (◇, ◆) 200 μg/ml (×) or 400 μg/ml (+). Pressure was set to 0.1, 50, 100, 150, 100, 50 and 0.1 MPa for two consecutive cycles and at each given pressure the polarization of fluorescence was determined at 20°C. One set of measurements was carried out in the absence of detergent (open symbols), and a parallel set in the presence of 0.1 mg $C_{12}E_8$/ml (filled symbols). The corresponding detergent to protein ratios were (mg/mg) 100 (▲), 20 (■), 2 (●), 1 (◆), 0.5 (×) or 0.25 (+).

The effect of vanadate

In a calcium-free medium containing 5 mM vanadate the polarization of fluorescence was relatively high ($P \cong 0.36$) even in the absence of detergents (Fig. 14B, circles). Under these conditions the Ca^{2+}-ATPase forms stable two-dimensional membrane crystals [53,54] containing ATPase dimers as structural units; the dimers are held together by massive protein bridges between the cytoplasmic domains of the Ca^{2+}-ATPase [55,56]. The increased polarization of fluorescence of FITC-Ca^{2+}-ATPase in the presence of vanadate suggests that the structure of the Ca^{2+}-ATPase in the crystalline arrays may be different from the structure of the naturally occurring ATPase oligomers in the native membrane. Addition of $C_{12}E_8$ (2 mg/mg protein) to these suspensions increased the polarization of fluorescence at atmospheric pressure to $P \cong 0.425$ (Fig.

14B; squares), consistent with the detergent-induced disruption of Ca^{2+}-ATPase crystals [53,54], and the formation of ATPase monomers [47]. The small reversible pressure induced increase at 150 MPa was not influenced by vanadate (Fig. 14B).

The absence of a causal relationship between the pressure-induced inhibition of ATPase activity and the dissociation of ATPase oligomers

The pressure dependence of ATP hydrolysis by native and $C_{12}E_8$-solubilized sarcoplasmic reticulum can be compared with the pressure dependence of the fluorescence polarization of FITC covalently bound to the Ca^{2+}-ATPase under similar experimental conditions.

Much of the pressure-induced inhibition of ATPase activity in native sarcoplasmic reticulum vesicles (Fig. 1; solid circles) occurred in a relatively low pressure range (50–100 MPa), where there was no significant dissociation of ATPase oligomers in FITC-labeled preparations (Fig. 14A). The inhibition of ATPase activity was entirely reversible upon releasing the pressure from 150 MPa to 0.1 MPa (Fig. 1; open circles). Reciprocally nearly complete dissociation of ATPase oligomers could be obtained at 0.1 MPa pressure in the presence of 2 mg $C_{12}E_8$/mg protein (Fig. 14A; squares), with only moderate inhibition of ATP hydrolysis (Fig. 1; squares). These observations provide additional evidence that the solubilized ATPase retains high ATP hydrolyzing activity [47], and that the pressure-induced inhibition of ATP hydrolysis is not caused by changes in ATPase–ATPase interactions.

Pressure-induced changes in the structure of the lipid phase of sarcoplasmic reticulum

The location of 6-propionyl-2-(dimethylamino) naphthalene (Prodan) in artificial lipid bilayers and in brain synaptic membranes is altered by pressure, causing changes in its fluorescence and infrared spectra [57,58]. Based on these observations Prodan was used as fluorescent probe to analyze the effects of pressure on the properties of the lipid phase in sarcoplasmic reticulum membranes (Figs. 16–18).

The fluorescence emission spectrum of free Prodan measured in the absence of sarcoplasmic reticulum is characterized by an emission maximum at ≅ 520–525 nm that shows a 2 nm reversible red shift at 150 MPa pressure in media containing 0.1 mM Ca^{2+} (Fig. 16). Essentially identical spectra were obtained in Ca^{2+}-free media containing 0.1 mM EGTA and 5 mM vanadate (Fig. 17). The small peak at 409 nm is the Raman band of water. There is no observable pressure dependence of the excitation spectra.

In suspensions of sarcoplasmic reticulum vesicles at atmospheric pressure the partitioning of Prodan into the lipid phase caused a shift of the main emission

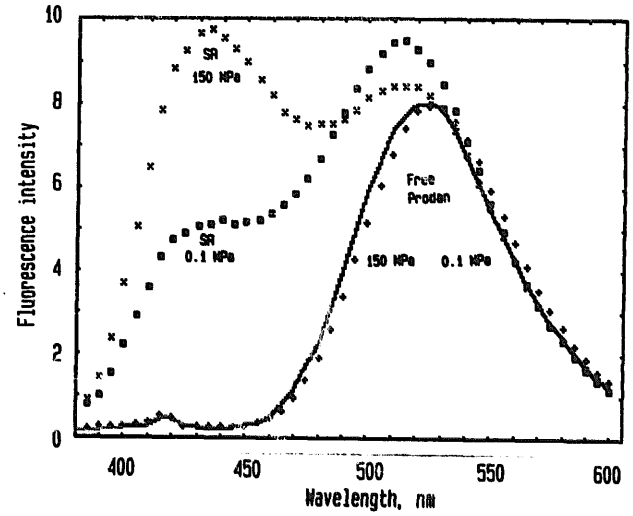

Fig. 16. Fluorescence emission spectra of Prodan in the presence of Ca^{2+}. The fluorescence of Prodan (0.5 μM) was measured at 380–600 nm using light of 360 nm for excitation, either in the absence of SR (−, +), or in the presence of 50 μg/ml SR (×, ■). Samples were preincubated for 30 min at 20°C at 0.1 MPa to allow temperature equilibration. Measurements were performed at 0.1 MPa (−, ■) or 150 MPa pressure (×, +), in a medium containing 0.1 M KCl, 20 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$ and 0.1 mM $CaCl_2$ to stabilize the E_1Ca_2 state of the Ca^{2+}-ATPase.

band from ≅ 520 to ≅ 510 nm and a new emission maximum appeared at ≅ 430 nm (Fig. 16). The emission band at 430 nm is attributed to membrane-bound dye in an environment of low polarity, while the band centered at ≅ 510–515 nm is the unresolved sum of the emission bands of the unbound dye and of the membrane-bound dye in a more polar environment

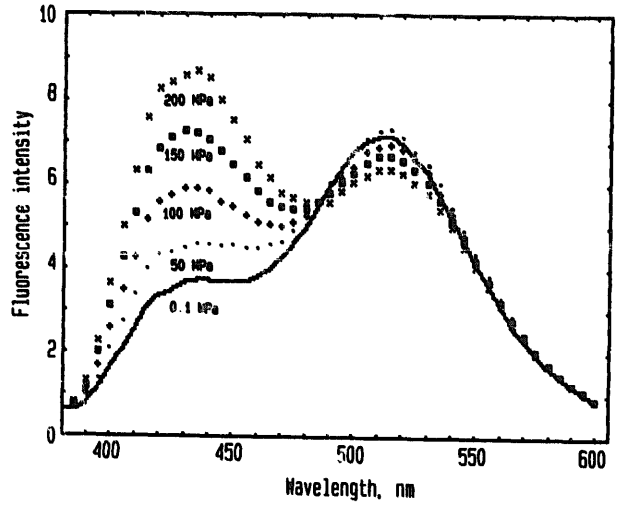

Fig. 17. Effect of pressure on the emission spectra of Prodan in a low-Ca^{2+} medium containing 5 mM vanadate. Fluorescence was excited at 360 nm (slit width, 4 nm) and the emission intensity recorded at the indicated wavelength at 20°C. Medium: 100 mM KCl, 5 mM $MgCl_2$; 20 mM Tris-HCl (pH 7.0)+0.5 μM Prodan+50 μg/ml sarcoplasmic reticulum. The enzyme was stabilized in the E_2 state by the addition of 0.1 mM EGTA+0.5 mM Na_3VO_4. Pressures: 0.1 MPa (solid line), 50 MPa (·····), 100 MPa (+), 150 MPa (■) and 200 MPa (×).

[56]. The ratio of the emission intensities at 430 and at 510 nm was similar in Ca^{2+}-containing media that stabilize the Ca_2E_1 state (Fig. 16) and in EGTA + vanadate containing media (Fig. 17) that stabilize the E_2V state of the Ca^{2+}-ATPase.

Increasing the pressure to 150 MPa nearly doubled the emission intensity at 430 nm with slight decrease of the emission at 510 nm either in the E_1 (Fig. 16) or in the E_2V states (Fig. 17). These observations indicate a pressure-induced redistribution of Prodan in sarcoplasmic reticulum, that is not affected significantly by the conformational state of the Ca^{2+}-ATPase. The increase in the intensity of fluorescence emission at 430 nm is already significant at 50–100 MPa pressure (Fig. 17), where the inhibition of the Ca^{2+}-stimulated ATP hydrolysis occurs (Fig. 1) and continues to develop nearly linearly with increase in pressure up to and probably beyond 200 MPa (Fig. 17).

The polarization of fluorescence of Prodan measured in the presence of sarcoplasmic reticulum was much higher at 435 nm (Fig. 18A) than at 510 nm (Fig. 18C). At 510 nm the polarization of fluorescence increased steadily up to 200 MPa and it was fully reversed after returning to 0.1 MPa pressure (Fig. 18C). Systems containing egg phosphatidylcholine showed qualitatively similar behavior (Fig. 18C). The polarization of fluorescence of free Prodan measured in the absence of sarcoplasmic reticulum was low (0.04–0.05), and changed only slightly with pressure (not shown). The similar pressure dependence of polarization at 510 nm in sarcoplasmic reticulum vesicles and in phosphatidylcholine liposomes is consistent with the suggestion that the 510 nm band arises from Prodan molecules located in the relatively polar phospholipid headgroup region of the membrane.

At 435 nm the pressure dependence of fluorescence polarization measured in the presence of sarcoplasmic reticulum was biphasic, with a steep increase in polarization up to 100 MPa, and decrease in polarization between 100–200 MPa pressure (Fig. 18A). The pressure-induced changes in polarization were largely reversible over the whole pressure range. Solubilization of the vesicles with $C_{12}E_8$ reduced the absolute value of polarization, but the biphasic pressure dependence was still evident, even at $C_{12}E_8$ concentrations as high as 10 mg/mg protein (Fig. 18A). In systems containing egg phosphatidylcholine the polarization of Prodan fluorescence measured at 435 nm reached a plateau between 100–200 MPa (Fig. 18B); the negative pressure dependence seen with sarcoplasmic reticulum in this pressure range (Fig. 18A) was not observed with phosphatidylcholine liposomes.

The increase in polarization at 435 nm up to $\cong$ 100 MPa pressure is consistent with redistribution of the naphthalene ring and the dimethylamino group of Prodan from the region of the phospholipid headgroups into the acyl chain domain of the membrane [57,58]. The decline in polarization at 200 MPa suggests that

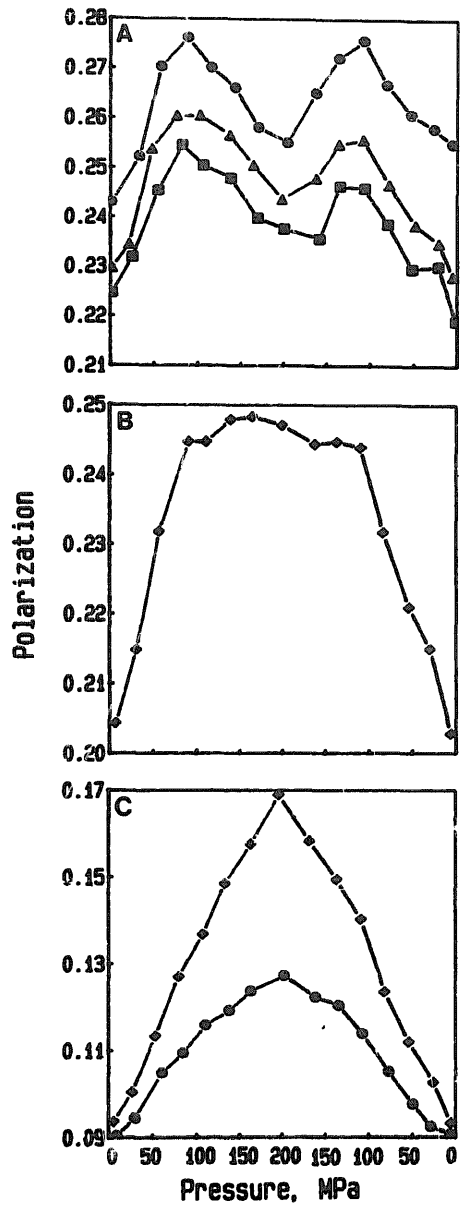

Fig. 18. The effect of pressure on the fluorescence polarization of Prodan in sarcoplasmic reticulum and in egg phosphatidylcholine liposomes. The polarization of fluorescence of Prodan (0.5 μM) was measured at 435 nm (A, B) and at 510 nm (C) using light of 365 nm for excitation. The samples contained 0.1 M KCl, 20 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 0.1 mM EGTA and either 50 μg sarcoplasmic reticulum protein/ml (●, ▼ ■) or 50 μg egg phosphatidylcholine per ml (◆). To samples marked ▲ and ■ 2 and 10 mg $C_{12}E_8$ per mg protein were added just before the measurements. The samples were preincubated at 20°C for 30 min, for temperature equilibration. The polarization of fluorescence was measured in T configuration during two consecutive pressure cycles from 0.1 MPa to 200 MPa and back to 0.1 MPa in steps of $\cong$ 28.5 MPa. Only the results of the first pressure cycle are shown. At any given pressure 5 min were allowed for the redistribution of the dye before the measurement was initiated. Each symbol represents the average of 15 consecutive polarization measurements with a standard error of mean less than 0.002. (A) Fluorescence polarization of Prodan measured in sarcoplasmic reticulum suspensions in the absence of detergent (●——●) or in the presence of 2 mg $C_{12}E_8$/mg SR protein (▲——▲), or 10 mg $C_{12}E_8$/mg SR protein (■——■) at an emission wavelength of 435 nm. (B) Fluorescence polarization of Prodan measured at 435 nm in the presence of egg phosphatidylcholine (◆). (C) Fluorescence polarization of Prodan in the presence of SR (●) or egg phosphatidylcholine (◆) at 510 nm.

Prodan is forced by pressure into an environment of low polarity, where its motional freedom is enhanced. As the biphasic pressure dependence of polarization was observed only on sarcoplasmic reticulum vesicles (Fig. 18A), but not in phosphatidylcholine liposomes (Fig. 18B), the region occupied by Prodan at 150–200 MPa pressure may be at the interface between lipids and proteins where the bilayer structure is expected to be less regular. The decrease in polarization at 150–200 MPa pressure is not likely to be associated with protein denaturation, since it is fully reversible.

Since the concentration of Prodan was only 1 mol/mol ATPase or 1 Prodan per ≅ 100 phospholipid molecule it is unlikely that Prodan itself would have significantly influenced the organization of the membrane.

Considering the dependence of Ca^{2+}-ATPase activity on the structure of membrane phospholipids [44] and the well known effects of pressure on the structure of phospholipid bilayers [2,19–22], it is plausible to assume that the pressure-induced changes in Prodan fluorescence may have relevance to the mechanism of pressure effects on ATPase activity.

Discussion

The reversible inhibition of Ca^{2+}-stimulated ATP hydrolysis at 50–100 MPa pressure indicates that the reaction has a positive activation volume. The activation volume is pressure-dependent, increasing from ≅ 20 ml/mol at atmospheric pressure to 53 ml/mol at 100 MPa (Fig. 1); somewhat higher values of 30–90 ml/mol were obtained for sarcoplasmic reticulum solubilized with $C_{12}E_8$. The pressure dependence of the activation volume implies that the rate-limiting step of ATP hydrolysis is different at low and high pressures.

Although the experimental activation volume is readily obtainable at saturating concentrations of Ca^{2+} and Mg-ATP from the relationship of ln k versus pressure, its unambiguous interpretation in terms of the complex reaction mechanism of the Ca^{2+}-ATPase remains elusive. This is due in part to continuing uncertainties about fundamental aspects of the reaction mechanism [36,59], but the main stumbling block is the complexity of the processes that participate in the volume changes [13,49]. Among the likely contributions to the activation volume are: (a) the orientation of water dipoles around charged or polar groups of protein; (b) the hydrophobic hydration; (c) the hydrogen bonded interactions of water molecules with each other and with the protein surface [13].

Each of these equilibria are perturbed by the binding of substrates to the enzyme and by the structural rearrangements during the catalytic process. The close association of the ATPase molecules with membrane phospholipids [44,60] and the protein-protein contacts

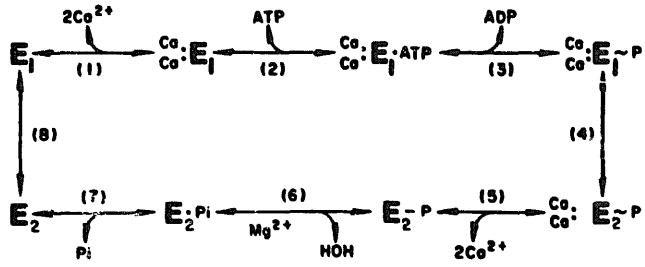

Fig. 19. Elementary steps of ATP hydrolysis.

involved in the formation of ATPase oligomers [61–63] are also likely components of the pressure-dependent volume changes.

The mechanism of ATP-dependent Ca^{2+} transport outlined in the scheme above (Fig. 19) postulates the existence of two major conformations of the Ca^{2+}-ATPase (E_1 and E_2) that alternate during the reaction cycle (Ref. 35; see, however, Ref. 59). The enzyme interacts with Ca^{2+} and Mg-ATP in the hypothetical E_1 conformation on the cytoplasmic surface and a covalent phosphorylated enzyme intermediate forms with the occlusion of Ca^{2+}. The translocation and release of calcium on the luminal side of the membrane is followed by the hydrolysis of phosphoenzyme intermediate. During reversal of the process the Ca^{2+}-free enzyme interacts with Mg and P_i in the hypothetical E_2 conformation forming a low-energy phosphoenzyme intermediate, that subsequently binds and translocates Ca^{2+} from the luminal to the cytoplasmic surface coupled to the synthesis of ATP [35].

The activation volumes measured at saturating substrate concentrations showed similar dependence on pressure with Mg-ATP (this report), Mg-*p*-nitrophenyl phosphate (PNPP) [8] and Mg-dinitrophenyl phosphate (DNPP) [9] as substrates ranging from ≅ 20 ml/mol at 0.1 MPa to ≅ 80 ml/mol at 100 MPa. At subsaturating substrate concentrations the pressure dependence of the reaction velocity remained essentially unchanged with both pseudosubstrates (PNPP and DNPP), yielding small binding volumes (≤ 20 ml/mol) at atmospheric pressure, and essentially zero binding volume at 100 MPa [8,9]. Similar experiments are difficult to perform accurately with Mg-ATP due to the high affinity of the binding site (K_m(ATP) ≅ 1–10 μM). Nevertheless, considering the identical activation volumes obtained with ATP, PNPP and DNPP as substrates, it is safe to assume that the binding of Mg-ATP, like that of other substrates, is not accompanied by major volume changes.

The data on the probable volume changes related to Ca^{2+}-binding are more ambiguous. In this report variations of the free Ca^{2+} concentration over a wide range altered only slightly the pressure dependence of ATP hydrolysis at saturating Mg-ATP concentration (Fig. 3), suggesting that the binding volume of Ca^{2+} was small.

By contrast, Hasselbach [9] observed a steep decline in the rate of *p*-nitrophenyl phosphate and dinitrophenyl phosphate hydrolysis with increasing pressure at subsaturating Ca^{2+} concentrations that yielded apparent Ca^{2+} binding volumes of 35–67 ml/mol at 0.1 MPa and 150–155 ml/mol at 100 MPa [8,9]. These volumes far exceeded the reaction volume of ≅ 20 ml/mol expected for the chelation of Ca^{2+}. These observations may imply that the binding volume of Ca^{2+} depends on the energy donor substrate. However, the possibility is not excluded that secondary processes, only indirectly related to the Ca^{2+} saturation of the ATPase, may have contributed in the earlier studies [8,9] to the large volume changes attributed to Ca^{2+} binding. ATP (5 mM) provides significant protection against irreversible inactivation of the Ca^{2+}-ATPase at 80–200 MPa pressure at low Ca^{2+} concentration, that is not seen with 5 mM *p*-nitrophenyl phosphate, acetyl phosphate or carbamyl phosphate [3,5]. Therefore the steep loss of PNPPase and DNPPase activities at 30–80 MPa pressure at subsaturating Ca^{2+} concentrations [9] may have included some irreversible inactivation of the Ca^{2+}-ATPase, and may not truly reflect the binding volume of Ca^{2+}.

The pressure-induced reversible inhibition of Ca^{2+}-stimulated ATPase activity occurred without significant change in the free Ca^{2+} concentration required for half maximal activation of ATP hydrolysis. These observations suggest that increasing pressure reversibly decreases the concentration of the Ca^{2+}-ATPase species that is capable to react with Ca^{2+} and ATP, without changing the Ca^{2+} affinity of the reactive enzyme form. Such behavior would be expected if at 50–100 MPa pressure the equilibrium between the E_1 and E_2 conformers of the Ca^{2+}-ATPase would shift from the Ca_2E_1 to the E_2 form, depleting the concentration of the E_1 species of high Ca^{2+} affinity. Several lines of indirect evidence support this explanation.

(1) The fluorescence intensity of FITC-labeled Ca^{2+}-ATPase suspended in Ca^{2+} containing medium that favors the Ca_2E state increased by ≅ 10–15% upon raising the pressure to 150 MPa (Figs. 4 and 5); this is consistent with the formation of the E_2 species of high fluorescence yield [39,40]. The fluorescence intensity of FITC-labeled Ca^{2+}-ATPase stabilized in the E_2V state by EGTA and vanadate was unaltered or slightly reduced at high pressure under otherwise identical conditions.

(2) Vanadate (1 mM) nearly completely inhibits the high affinity Ca^{2+} binding to the Ca^{2+}-ATPase [64]. The Ca^{2+} binding to sarcoplasmic reticulum measured with the fluorescent Ca^{2+} indicator Fluo-3 was reduced near the level of vanadate-treated Ca^{2+}-ATPase by raising the pressure to 100 MPa. This indicates essentially complete conversion of the Ca^{2+}-ATPase into a form that cannot bind Ca^{2+} with high affinity.

(3) At 100 MPa pressure where the Ca^{2+}-stimulated ATP hydrolysis was nearly completely inhibited, the enhancement of TNP-AMP fluorescence by 100 mM P_i in the absence of Ca^{2+} was essentially unaffected at pH 6.0 and was only slightly reduced at pH 7.0 or 8.0 (Fig. 12). As this reaction is attributed to phosphorylation of the enzyme by P_i in the E_2 state [45], the reactivity of the enzyme with P_i in the E_2 state is largely preserved at 100 MPa pressure. These observations also imply that the binding of TNP-AMP is relatively insensitive to pressure, consistent with the small pressure dependence of the binding of energy donor substrates. A secondary effect of pressure that is not explainable by the stabilization of the E_2 state, is the strong inhibition of the P_i-induced TNP-AMP fluorescence at 50–100 MPa pressure in the presence of 30 mM P_i at pH 7 and 8 (Fig. 11).

(4) Stabilization of the Ca^{2+}-ATPase in the E_2V conformation by vanadate or in the E_2Pi conformation by inorganic phosphate protected the enzyme from irreversible inactivation at 200 MPa pressure, where rapid denaturation occurs in the Ca_2E_1 state [5]. The protection by vanadate or Pi suggests that the molecular volume of the Ca^{2+}-ATPase is smaller in the E_2V or E_2Pi states than in the Ca_2E_1 state. Such volume difference would explain the trapping of the Ca^{2+}-ATPase in the E_2 conformation at high pressure.

Although these observations are consistent with the pressure dependence of the $E_1 \rightleftharpoons E_2$ equilibrium, other explanations are certainly not excluded. The inhibition of the P_i-induced enhancement of TNP-AMP fluorescence at 100 MPa pressure at pH 7 and 8 (Figs. 11 and 12), and the absence of any clear relationship between the predicted conformation of the Ca^{2+}-ATPase and the effect of pressure on the tryptophan fluorescence of sarcoplasmic reticulum (Fig. 6) are the two main observations that are difficult to reconcile with a pressure-dependent shift in the $E_1 \rightleftarrows E_2$ equilibrium.

It remains to be determined whether these observations represent secondary changes that coexist with pressure-induced $E_1 \rightarrow E_2$ transition or primary effects of pressure that alter the Ca^{2+} and substrate binding sites of the Ca^{2+}-ATPase without change in the $E_1 \rightarrow E_2$ equilibrium.

Pressure-dependent changes in the lipid phase of sarcoplasmic reticulum

Compression of the lipid bilayer at high pressure enhances the molecular order of the membrane phospholipids and increases the gel liquid crystalline phase transition temperature by ≅ 17–22 K/100 MPa [19–22]. Since the Ca^{2+}-ATPase is dependent on a fluid lipid environment for activity [60,65,66], the modification of the structure and dynamics of the lipid phase at high pressure is expected to influence the ATPase activity. Indeed Heremans and Wuytack [2] found that

a break in the Arrhenius plot of ATPase activity, that is located at 18°C at 0.1 MPa increases by 27 K/100 MPa increase in pressure. Therefore the Ca^{2+}-ATPase subjected to 100 MPa pressure at 20–25°C would experience an environment similar to near 0°C at atmospheric pressure.

The fluorescence emission spectrum of Prodan in buffer solutions has a maximum near 525 nm, consistent with the high polarity of the medium (Figs. 16 and 17); in sarcoplasmic reticulum vesicles the emission of Prodan had two local maxima of near 510 and 430 nm. The 510 nm emission arises from the contributions of the unbound Prodan and from Prodan located at a relatively polar site within the bilayer, while the 430 nm emission indicates a more hydrophobic environment [57,58]. Upon increasing the hydrostatic pressure the 430/510 ratio increased nearly linearly with pressure up to and probably beyond 200 MPa (Fig. 18), indicating relocation of Prodan to a more hydrophobic environment. The presence of an isoemissive point ≅ 480 nm is consistent with an interchange between two species of Prodan molecules. The increase in the 430/510 fluorescence intensity ratio between 50 and 100 MPa was accompanied by inhibition of ATPase activity.

The physical basis of the preference for the less polar environment at high pressure is unknown, but it may be related to the pressure-induced redistribution of Ca^{2+}-ATPase between Ca^{2+} sensitive (E_1) and Ca^{2+} insensitive (E_2) forms. Deeper immersion of Ca^{2+}-ATPase into the membrane bilayer was observed by freeze-etch electron microscopy of the E_2V type Ca^{2+}-ATPase crystals [67] and by X-ray and neutron diffraction analysis of sarcoplasmic reticulum membranes actively transporting Ca^{2+} [68].

Effect of pressure on ATPase–ATPase interactions

The existence of Ca^{2+}-ATPase oligomers in sarcoplasmic reticulum membranes is well established [60–63] but there is considerable disagreement about the stability of ATPase–ATPase interactions in detergent solutions and their functional significance [6,47]. An increase in hydrostatic pressure promotes the dissociation of ATPase oligomers solubilized in $C_{12}E_8$, as indicated by the change in the polarization of the intrinsic tryptophan fluorescence and of the fluorescence of pyrene maleimide covalently attached to the Ca^{2+}-ATPase [6].

Different two-dimensional crystal forms of the Ca^{2+}-ATPase are induced by Ca^{2+} or lanthanides in the E_1 state [51,69–71] and by vanadate in the E_2V state [53–56]. This raises the possibility that the ATPase oligomers are stabilized by different types of interactions, depending on their conformation.

Much of the pressure-induced inhibition of ATPase activity in native sarcoplasmic reticulum vesicles occurred at relatively low pressures (midpoint pressure ≅ 40 MPa) where there was no significant change in the polarization of fluorescence of FITC-ATPase, suggesting little or no change in the oligomeric structure of the Ca^{2+}-ATPase (Figs. 13–15). Therefore dissociation of ATPase oligomers cannot contribute significantly to the activation volume of ATP hydrolysis. Furthermore, complete dissociation of ATPase oligomers could be induced in the presence of 10 mg $C_{12}E_8$/mg protein at atmospheric pressure, without significant inhibition of ATPase activity (Figs. 13 and 15), and the pressure dependence of the ATPase activity of solubilized sarcoplasmic reticulum was similar to that of the native membranes (Fig. 1).

The Ca^{2+}-ATPase oligomers are stabilized by 2–20 mM Ca^{2+} against dissociation at 0.1 MPa pressure at high detergent: protein ratio (10 mg $C_{12}E_8$/mg protein) or at 150 MPa pressure at moderate detergent: protein ratios (2 mg $C_{12}E_8$/mg protein) (Fig. 13); this effect may contribute to the protection of Ca^{2+}-ATPase against irreversible inactivation at 150 MPa pressure in both native and detergent-solubilized preparations [5,12].

Deep sea fishes are continually exposed to 40–50 MPa pressure and low temperatures (2–3°C) in their natural environment [72,73]. The rate of muscle relaxation in these animals would be very slow if compensatory mechanisms would not exist to enhance the Ca^{2+} transport capacity of sarcoplasmic reticulum inhibited by the high pressure and low temperature. The compensation may involve an increase in the sarcoplasmic reticulum content of the muscle or the production of Ca^{2+}-ATPase isoenzymes that are specially adapted to function at low temperature and high pressure. Pressure activation of several enzymes has in fact been observed [1,13] and in a barophilic bacterium the expression of the ompH protein is activated by 28 MPa hydrostatic pressure [74]. Under physiological conditions the Ca^{2+} concentration in the lumen of the sarcoplasmic reticulum of resting muscle is ≅ 10–20 mM; the high luminal Ca^{2+} concentration may play a role in the stabilization of Ca^{2+}-ATPase against irreversible inactivation by prolonged exposure to high pressure.

Acknowledgements

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