Effect of Temperature on Ca-ATPase from Sarcoplasmic Reticulum Membranes: ESR Studies

A. M. RUBTSOV*, M. ŠENTJURC and M. SCHARA

J. Stefan Institute, E. Kardelj University of Ljubljana, Ljubljana 61 000, Jamova 39, Yugoslavia

Abstract. Using spin-labeled fatty acid derivatives and maleimide, the effect of temperature on the structural state of various parts of the lipid bilayer of sarcoplasmic reticulum (SR) membranes and the segmental motion of the Ca-ATPase molecule were investigated. The mobility of the spin probes localized in the hydrophobic zone and the outer part of the SR membrane was shown to increase with a rise in temperature from 4 to 44 °C, the temperature of 20 °C being critical for these changes. In the presence of ATP, critical changes in the spin probe mobility occur at lower temperatures, while in the presence of ATP and Ca^{2+} they are observed at 20 °C for a spin probe localized in the outer part of the SR membrane. The mobility of a spin probe localized in the hydrophobic part of the membrane increases linearly with a rise in temperature. In the absence of ligands, the segmental motion of Ca-ATPase changes linearly within a temperature range of 10—30°C. However, when ATP alone or ATP and Ca^{2+} are simultaneously added to the incubation mixture, the protein mobility undergoes critical changes at 20 °C. The Arrhenius plots for ATPase activity and Ca^{2+} uptake rate in SR membrane preparations also have a break at 20 °C. It is assumed that changes in the structural state of membrane lipids produce conformational changes in the Ca-ATPase molecule; the enzyme seems to be unsensitive to the structural state of the membrane lipid matrix in the absence of the ligands.

Key words: Sarcoplasmic reticulum — Ca-ATPase — ESR studies — Structural state of lipids — Segmental motion of protein

* Present address: Department of Biochemistry, School of Biology, M. V. Lomonosov Moscow State University, Moscow 119899, USSR

Reprint requests: Prof. M. Schara, J. Stefan Institute, E. Kardelj University of Ljubljana, Jamova 39, 61 000 Ljubljana, Yugoslavia

Introduction

The analysis of temperature dependence of various biochemical reactions is a useful tool in the study of living systems. Although "in vivo" enzymatic complexes operate under relatively constant temperature, temperature modifications "in vitro" may give valuable information on the structure, properties, and mechanism of action of a particular enzyme or an enzyme system. Assuming that temperature variations can influence significantly the structural state of the membrane lipid phase and, accordingly, of membrane intrinsic proteins, the analysis of temperature dependence seems to be the most promising approach (Pringl and Chapman 1981). This method has been widely used for the study of membrane enzymes, such as transport ATPases, e.g., Ca-ATPase (Boldyrev et al. 1976; Inesi et al. 1973; Boldyrev 1981). It was shown, in particular, that the Arrhenius plots for the temperature dependence of transport ATPase activity are not described by one line, since the experimental curve norr ally consists of two straight lines with a break around 20 °C (Inesi et al. 1973; Kirino et al. 1981). The reasons for the appearance of such a break in the Arrhenius plots have been extensively discussed in the current literature. According to some authors, at temperatures around 20 °C the protein conformation and, accordingly, the enzyme activity undergo certain changes (Kirino et al. 1981; Dean and Tanford 1978). Other investigators believe that the structural rearrangements in the membrane lipid phase induce thermotropic changes in the transport ATPase activity (Boldyrev et al., 1976; Quinn 1981). To elucidate this question, various physico-chemical methods have been employed, e.g., fluorescence measurements, calorimetric assays and ESR spectroscopy (Inesi et al. 1973; Boldyrev 1983). However, the results obtained through the use of these techniques have so far failed to provide for an unequivocal interpretation of the role of protein and lipid components in the thermoinduced changes of transport ATPase activity.

The aim of the present work was to study the changes in the state of the Ca-ATPase molecule and in the lipid phase of the sarcoplasmic reticulum (SR) membrane under various experimental conditions, using spin probes of different nature. The study included an analysis of temperature dependence of the parameters which characterize the mobility of two membrane incorporated fatty acid spin probes carrying a nitroxyl radical in different parts of the molecule and thus reflecting the state of different zones of the SR membrane as well as the mobility of the maleimide spin label covalently linked to the SH-groups of Ca-ATPase. A correlation was established between the changes in the structural state of the membrane lipid bilayer and the segmental motion of the Ca-ATPase molecule in the presence of ligands (ATP or ATP and Ca^{2+}).

Materials and Methods

SR membranes were isolated from rabbit white skeletal muscles by the differential centrifugation method (Rubtsov 1982). The Ca-ATPase activity and rate of Ca^{2+} uptake in SR membrane preparations were measured potentiometrically (Bessonov et al. 1974) in a medium containing 100 mmol/l KCl, 5 mmol/l $MgCl_2$, 3 mmol/l ATP, 5 mmol/l potassium oxalate, 3 mmol/l imidazole (pH 7.0), and 0.05—0.1 mg/ml of SR protein. The time of Ca^{2+} uptake was determined during 2—3 additions of $CaCl_2$ (50—100 nmol). For the titration a standard solution of KH_2PO_4 was used.

To label the SR proteins with N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide (MiSL) the SR vesicles, at a concentration of 10 mg protein/ml, were incubated overnight at 4 °C with 10 mmol/l MiSL in a solution containing 100 mmol/l KCl, 0.5 mmol/l EGTA, 3 mmol/l ATP and 20 mmol/l histidine (pH 7.0). To remove the nonreacted label and ATP, the suspension of SR vesicles after incubation was diluted 25 times with ice cold solution containing 100 mmol/l KCl and 20 mmol/l histidine (pH 7.0) and the sample was centrifuged at 100,000 × *g* 30 min. The pellet was resuspended in 0.3 mol/l sucrose and 20 mmol/l histidine (pH 7.0) to a protein concentration of 40 mg/mol. The MiSL-labeled SR preparations were measured in solution containing 100 mmol/l KCl, 0.25 mol/l sucrose, 0.8 mmol/l EGTA and 20 mmol/l histidine (pH 7.0) in the absence or presence of 1 mmol/l $CaCl_2$ and/or 3 mmol/l ATP.

To label membranes with fatty acid spin probes, 1-oxyl-4, 4-dimethyl-2-undecyl-2-(3-carboxypropyl)-oxazolidine (HFASL(10,3)) or 1-oxyl-4, 4-dimethyl-2-propyl-2(11-methoxycarbonylundecyl)--oxazolidine (MeFASL(2,11)), the SR vesicles were incubated at a concentration of 20 mg of protein/ml with 2×10^{-5} mmol/l of the corresponding spin label in medium containing 100 mmol/l histidine (pH 7.0) in the absence or presence of 1 mmol/l $CaCl_2$ and or 3 mmol/l ATP. To denaturate the SR proteins the samples were heated to 60 °C for 20 min.

The samples were measured in glass capillaries on a Varian E-9 X-band spectrometer; modulation amplitude 0.2 mT, microwave power 20 mW. The temperature was varied within 4—60 °C, using a variable temperature controller.

Results

Effects of temperature on Ca-ATPase activity and rate of Ca^{2+} uptake by SR membranes

The preparations of light SR were obtained from rabbit hind limb white skeletal muscles by differential centrifugation as described previously (Rubtsov 1982). The enzyme preparations had a high Ca-ATPase content (above 80 %) and a relatively low content of Ca^{2+} binding proteins and other minor protein components of SR. The Arrhenius plots for the Ca-ATPase activity and the rate of Ca^{2+} uptake by the enzyme preparations both had breaks around 20 °C (Fig. 1). The value of activation energy for this reaction was about 125 kJ/mol at 5—20 °C and about 75 kJ/mol at 20—35 °C. These results agree well with the literature data (Inesi et al. 1973; Boldyrev et al. 1976), and suggest that at 20 °C the SR membrane Ca-pump undergoes critical changes.

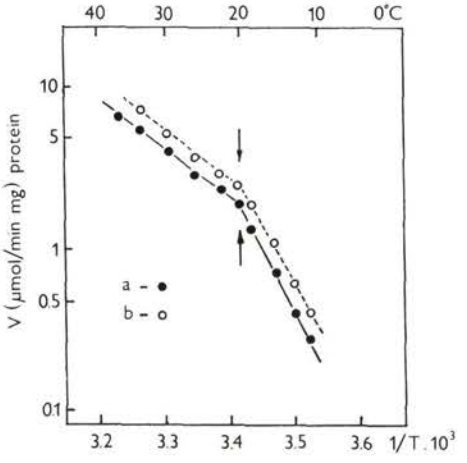

Fig. 1. The Arrhenius plots for Ca-ATPase activity (*a*) and Ca^{2+} uptake rate (*b*) in SR membrane preparations.

Study of SR membranes, using spin probes

Spin probes HFASL (10,3) and MeFASL (2,11) used in our study were taken at a concentration of 2×10^{-5} mol/l, which corresponds to 1 mole of spin-labelled fatty acid per ca. 200 moles of SR membrane phospholipids. Under these conditions, the spin probe molecules do not significantly affect the state and properties of the SR membrane lipid phase.

The spectra of MeFASL(2,11), a probe which gets dissolved in phospholipid bilayer, indicate a highly fluid environment of the nitroxide group (Fig. 2). The value of isotropic hyperfine splitting is about 1.37 mT at 20 °C, which points to the localization region of the SR membranes. The plot for the temperature dependence

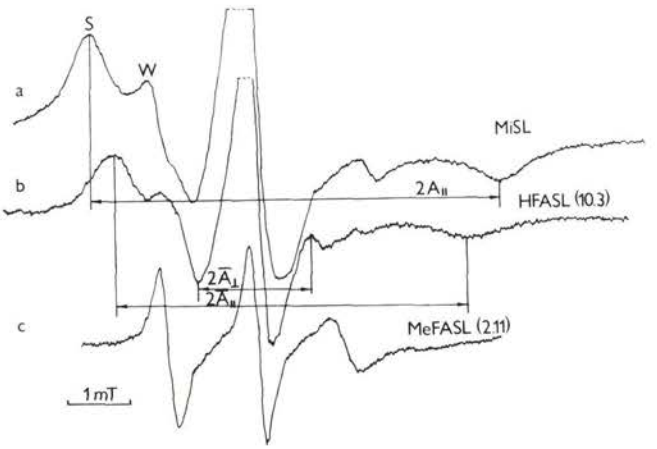

Fig. 2. ESR spectra of spin labeled SR preparations. (*a*) Labeled with MiSL, (*b*) Labeled with HFASL (10,3), (*c*) Labeled with MeFASL (2,11) at $t° = 20°C$.

of the empirical rotational correalation time has a break at 20—21 °C under control conditions (Fig. 3). Here τ is the empirical rotational correlation time determined from the ESR line width (Marsh 1981). In the presence of 3 mmol/l ATP, this break is shifted towards 15 °C. After addition of Ca^{2+} to the ATP-containing incubation mixture, in which Ca-ATPase is expected to be in a phosphorylated state, no break was observed in the ln τ against $1/T$ plot (Fig. 3).

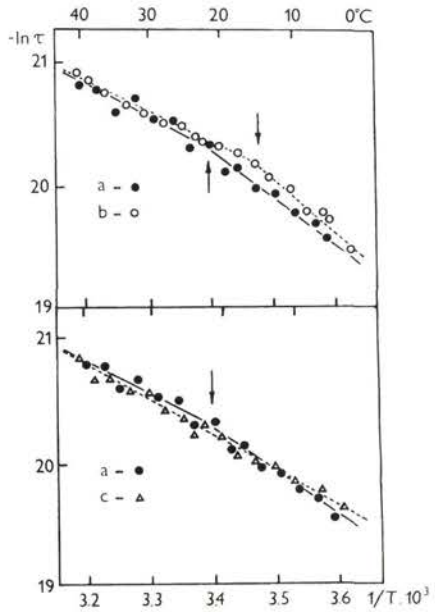

Fig. 3. Variation of the rotational correlation time with temperature for MeFASL (2,11) in SR membrane preparations. (*a*) Under control conditions, (*b*) In the presence of 3 mmol/l ATP, (*c*) In the presence of 3 mmol/l ATP and 1 mmol/l Ca^{2+}.

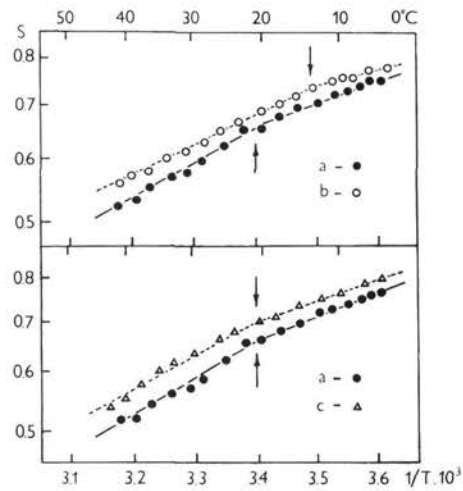

Fig. 4. Variation of the order parameter S with temperature of HFASL (10,3) in SR preparations. (*a*) In the absence of the ligands, (*b*) In the presence of 3 mmol/l ATP, (*c*) In the presence of 3 mmol/l ATP and 1 mmol/l Ca^{2+}.

In experiments with spin probe HFASL (10,3), which reflects the membrane phospholipid chain motion, the temperature variation of the order parameter S, calculated from the relation:

$$S = \varkappa(2\bar{A}_{\parallel} - 2\bar{A}_{\perp})/(2A_{\parallel} - 2A_{\perp}),$$

was used. Here $\bar{A}_{\parallel}$ and $\bar{A}_{\perp}$ are experimentally obtained components of averaged hyperfine splitting A (Fig. 2), while $A_{\parallel}$ and $A_{\perp}$ are the corresponding components of the hyperfine splitting tensor for the spin probe in a rigid environment. For HFASL (10,3) $A_{\parallel} = 3.36$ mT and $A_{\perp} = 0.605$ mT (Marsh 1981).

From the isotropic hyperfine splitting $a = \mathrm{Tr}A/3 = 1.53$ mT, it can be concluded that the polarity of the nitroxide group environment is nearly the same for the membrane as well as for buffer dissolved spin probe, where $a = 1.56$ mT was measured.

The plot for the order parameter S versus $1/T$ has a break at 20 °C; the rate of mobility alterations of the probe with temperatures above 20 °C is larger than that below 20 °C (Fig. 4). In the presence of 3 mmol/l ATP, the spin probe mobility is more restricted than in the control; under these conditions, the break in the S versus $1/T$ plot is shifted towards the low temperature region. Upon simultaneous addition of Ca^{2+} and ATP to the incubation mixture the dependence of parameter S on $1/T$ becomes similar to that of the control; however, at all temperatures studied the value of the order parameter S increases, thus suggesting a highly ordered microenvironment of the spin probe HFASL(10,3) under the experimental conditions given (Fig. 4).

Effects of temperature on the segmental motion of Ca-ATPase

In labeled SR vesicles about 10^{15} bound molecules of MiSL per mg of protein was measured from the ESR spectra intensity; the Ca-ATPase activity is retained to 70 %. The light preparations of SR used in the present study were characterized by a high Ca-ATPase protein content and a low content of other protein components of SR, including the glycoprotein with M_r 30,000 which, as shown previously, also contains SH-groups and can be also labeled with MiSL (Hidalgo and Thomas 1977). The low glycoprotein content in our preparations of SR and the type of the ESR spectra for the MiSL-treated SR membranes indicate that this spin label is predominantly bound to SH-groups of the Ca-ATPase protein and, accordingly, the spectra of this label reflect the state of the enzyme in the membrane.

ESR spectra (Fig. 2) show that the nitroxide groups of MiSL molecules are strongly restricted in their motion in the temperature range measured; thus indicating that SH-groups which bind the maleimide group of MiSL are not on the surface of the membrane or protein.

The plot for the dependence of the MiSL ESR spectrum parameter $2\bar{A}_{\parallel}$ that characterizes the segmental motion of the protein on $1/T$ is practically linear in the absence of the ligands within a temperature range of 10—30 °C (Fig. 5). Under these conditions, two breaks are observed at 7 °C and 30 °C, respectively. In the presence of ATP, a new break appear in the parameter $2A_{\parallel}$ versus $1/T$ plot at 20 °C. When ATP and Ca^{2+} are simultaneously added to the incubation mixture, the temperature dependence of the parameter $2\bar{A}_{\parallel}$ has only one break at 20 °C.

The same results were obtained by measuring the empirical disorder parameter W/S which reflects the state and the mobility of the proteins in the membranes (Hidalgo and Thomas 1977; Barber et al. 1983). According to Hidalgo and Thomas (1977), the ratio of the "weakly" and "strongly" immobilized components

of ESR spectra of MiSL-labeled SR preparations and purified Ca-ATPase is 0.40 and 0.17 respectively at 4 °C. Under our conditions, the ratio W/S was 0.29, 0.28 and 0.35 in the absence of the ligands and in the presence of ATP or ATP plus Ca^{2+}, respectively, at 4 °C (Fig. 6); this indicates a level of MiSL incorporation into Ca-ATPase protein. The temperature dependence of W/S was linear in the absence of the ligands within a temperature range of 4—35 °C (Fig. 6). In the presence of ATP, a break was observed at 18 °C. When ATP and Ca^{2+} were added into reaction medium, the W/S versus $1/T$ plot was nonlinear with a break at 25 °C.

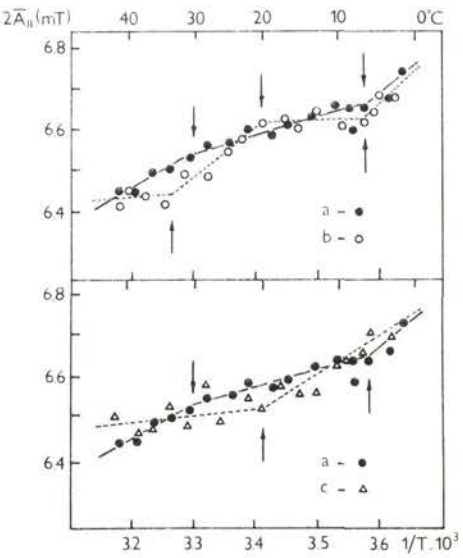

Fig. 5. Temperature dependence of the hyperfine splitting $2\bar{A}_{\parallel}$ of MiSL in SR preparations. (*a*) Under control conditions, (*b*) In the presence of 3 mmol/l ATP, (*c*) In the presence of 3 mmol/l ATP and 1 mmol/l Ca^{2+}.

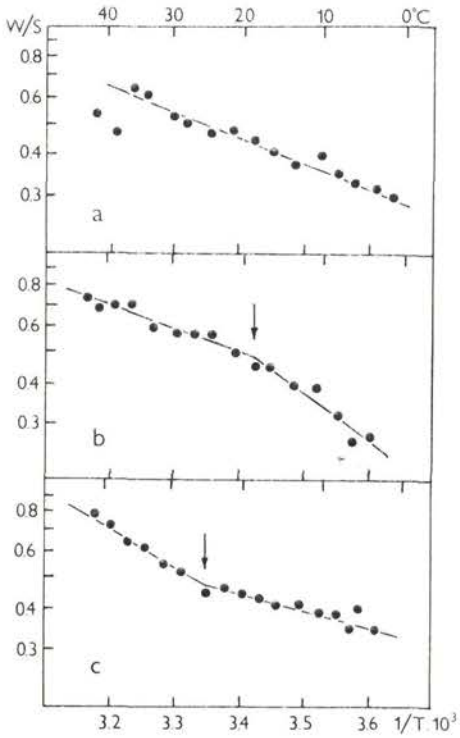

Fig. 6. Temperature dependence of the disorder parameter W/S for MiSL-labeled SR preparations. (*a*) In the absence of the ligands, (*b*) In the presence of 3 mmol/l ATP, (*c*) In the presence of 3 mmol/l ATP and 1 mmol/l Ca^{2+}.

Discussion

The fact that the Arrhenius plots for the temperature dependence of the Ca-ATPase activity and for the rate of Ca^{2+} uptake in light SR preparations have breaks at

20 °C suggests the existence of thermoinduced changes in the enzyme function (Fig. 1). Similar results were reported for crude SR membranes (Inesi et al. 1973) as well as for purified Ca-ATPase (Boldyrev et al. 1976). Consequently, Ca-ATPase undergoes significant changes; at 20 °C these may result from processes of a mixed nature, e.g., conformational shifts in the enzyme molecule, changes in protein-protein and protein-lipid interactions in the SR membrane, etc. (Boldyrev 1983).

The activity of Ca-ATPase, an intrinsic membrane protein whose function requires certain hydrophobic microenvironment, is predominantly controlled by the stage of the membrane lipid phase. Changes in the structural state of the SR membrane lipid phase and their effects on the functional activity of Ca-ATPase have been studied by many investigators; however, up till now there has been no unequivocal answer to this question. For instance, no correlation has been found so far between the temperature dependence of the Ca-ATPase activity characterized by a break at 20 °C and the behaviour in the SR membrane of the hydrophobic fluorescent probe diphenylhexatriene. The Arrhenius plot for the temperature dependence of fluorescence polarization for this probe is linear at 10—40 °C (Boldyrev 1983; Boldyrev et al. 1983). No critical changes in the SR membrane have been established for parameter $2\bar{A}_{\parallel}$ of the ESR spectrum for the spin-labeled fatty acid C_{18} at 20 °C whereas the temperature dependence of this parameter for the same fatty acid covalently linked to the Ca-ATPase protein is nonlinear with a break at 20 °C (Kirino et al. 1981). A break at 20 °C was observed in the Arrhenius plots for the ATPase activity when Ca-ATPase preparations, in which the lipids were substituted for by nonionic detergents, were used (Dean and Tanford 1978). This led some authors to question the key role of lipids in the thermoinduced changes of the Ca-ATPase activity and to assume that temperature (20 °C) effects the conformation of Ca-ATPase and, correspondingly, its functional properties (Dean and Tanford 1978; Kirino et al. 1981).

In our studies including the use of fatty acid spin probes MeFASL(2,11) and HFASL(10,3), it was shown that the temperature dependences of parameters τ and S for these spin probes in SR membranes are characterized by breaks around 20 °C (Figs. 3 and 4). These data suggest that the SR membrane lipid phase at 20 °C undergoes certain structural changes which affect both the inner and outer regions of the membrane. Similar results based on various physico-chemical approaches were reported in the literature. Studies with the use of some spin-labeled fatty acids have shown that the shifts in the structural state of membrane lipids are correlated with the thermoinduced changes in the Ca-ATPase activity of these preparations (Inesi et al. 1973). Similar modulations in the state of the SR membrane lipid phase were observed in experiments aimed at determining the temperature dependence of the degrees of eximerization of the fluorescent probe pyrene. This latter fluorescent probe is localized in the hydrophobic zone of the

membrane and characterizes the "hydrophobic volume of the membrane" (Boldyrev et al. 1982). The plot for the temperature dependence of the eximerization degree of pyrene is nonlinear and has a break at 20 °C. Moreover, the so-called resonance energy transfer from the tryptophane residues of Ca-ATPase to pyrene dissolved in the SR membrane is also subjected to critical changes at 20 °C (Boldyrev et al. 1983). This may suggest that these temperature changes not only affect the structural state of the membrane lipid bilayer but the mode of protein-lipid interaction as well. Hence, thermoinduced structural shifts in the membrane lipid phase may affect the function of Ca-ATPase. The differences observed in the experimental data obtained by different authors may be due to modifications in experimental conditions as well as to the nature of the enzyme preparations used (Boldyrev 1983).

After addition of 3 mmol/l ATP to the incubation medium, the break in the τ and S versus $1/T$ plots for spin probes MeFASL(2,11) and HFASL(10,3), respectively, is shifted towards a temperature region around 15 °C (Figs. 3 and 4). The ordering of the membrane layers close to the membrane surfaces at all temperatures used is slightly increased, whereas the mobility of the lipid hydrocarbon chains increases, on the contrary within a temperature range of 5—25 °C. As shown previously, the interaction between ATP and Ca-ATPase induces significant conformational changes in the enzyme molecule (Rubtsov et al. 1982; Rubtsov 1982). Moreover, ATP controls the protein-protein interactions in Ca-ATPase membranes by loosening intramolecular cross-links in the oligomeric complexes (Rubtsov 1982; Boldyrev et al. 1983). It is highly probable that the ATP-induced conformational changes in Ca-ATPase involve the lipid phase; this is reflected in changes in the state of different parts of the SR membrane. Addition of Ca^{2+} to incubation medium containing ATP induces phosphorylation of Ca-ATPase. The dependence of parameter τ on $1/T$ for spin probe MeFASL(2,11) is practically linear. The ordering of the outer membrane part of SR increases under these conditions, whereas the dependence of parameter S on $1/T$ remains nonlinear with a break at 20 °C. Assuming that Ca-ATPase phosphorylation is accompanied by significant changes in the conformation of the enzyme molecule (Rubtsov 1982) it may be supposed that the protein exerts an "orderly" influence on the polar regions of the SR membrane virtually without affecting the state of the inner hydrophobic part of the membrane.

An analysis of temperature effects on the segmenal motion of the Ca-ATPase molecule covalently linked to MiSL has shown that, within a temperature range of 10—30 °C, the label mobility increases linearly (Fig. 5), showing discontinuities only around 7 °C and 30 °C. The temperature dependence of the disorder parameter W/S is in fact also linear within all the temperature ranges studied (Fig. 6). Studies with intrinsic fluorescence of tryptophane residues in the Ca-ATPase molecule have shown that the fluorescence intensity linearly decreases

as the temperature rises (Boldyrev 1983; Boldyrev et al. 1983). This observation allowed the authors to rule out the occurrence of significant thermoinduced shifts in the hydrophobic parts of the enzyme molecule containing tryptophane residues. Nevertheless, the Arrhenius plots for the dependence of the Ca-ATPase activity on temperature in preparations of SR membranes have breaks at 20 °C.

In the presence of ATP, a new break appears on the plot for parameter $2\bar{A}_{\parallel}$ versus $1/T$ at 20 °C (Fig. 5). The temperature dependence of this parameter is also nonlinear in the presence of ATP and Ca^{2+} with a single break at 20 °C. The Arrhenius plots for the disorder parameter W/S for MiSL-labeled SR proteins are characterized by breaks at 18 °C and 25 °C in the presence of ATP or ATP and Ca^{2+}, respectively (Fig. 6). Hence, in the presence of the ligands, there is a correlation between breaks on the Arrhenius plots for MiSL-labeled SR proteins and those on the Arrhenius plots for the mobility of the spin probes MeFA-SL(2,11) and/or HFASL(10,3).

In previous experiments it was shown that the binding of ATP and Ca-ATPase phosphorylation result in considerable modulations in the enzyme molecule conformation (Rubtsov 1982; Rubtsov et al. 1982); under these conditions the protein has a "structural" influence on the outer regions of the SR membrane. Under these conditions only changes in the structural state of membrane lipids, or the outer parts of the lipid bilayer at least, are correlated with the segmental motion and the mobility of the Ca-ATPase molecule. Thus, the experimental results suggest that both the break in the Arrhenius plots for Ca-ATPase activity and the rate of Ca^{2+} uptake are due to the high sensitivity of Ca-ATPase in the presence of ATP, and especially of the phosphorylated enzyme, to structural transitions in the lipid matrix of the SR membranes.

According to the present-day concepts of the mechanism of Ca-ATPase function, the translocation of Ca^{2+} across the SR membrane takes place during the translocation of the phosphorylated enzyme from conformational state $E_1 \cdot 2Ca$ to conformational state $E_2 \cdot 2Ca$ (Moller et al. 1982). This process is accompanied by changes in the enzyme affinity for Ca^{2+}, as a result of which Ca^{2+} is released into the inner volume of SR. In this way, in the course of the phosphoenzyme conversions Ca-ATPase undergoes essential conformational changes that are necessary for the transmembrane translocation of Ca^{2+}. These results are well consistent with the hypothesis, according to which the above-mentioned conformational changes determine the high sensitivity of Ca-ATPase to the structural state of the SR membrane lipid phase. This circumstance may serve as a satisfactory explanation of the nonlinearity of the Arrhenius plots for Ca-ATPase activity and the rate of Ca^{2+} transport.

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