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Sarcoplasmic reticulum from rabbit and winter flounder: temperature-dependence of protein conformation and lipid motion

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A comparative study of lipids and proteins in sarcoplasmic reticulum (SR) from rabbit and flounder has been undertaken. The protein/phospholipid ratio (w/w) was 3:1 in flounder SR (FSR) and 2.2:1 in rabbit SR (RSR). Both membranes had similar contents of PC (70%) and PI (6%). PE constituted 15% in RSR and 21% in FSR. PS and sphingomyelin were minor components of both SR (< 4%). There were differences in the unsaturated chains of the total lipid extracts, PC, PE, and PI between FSR and RSR. RSR was high in linoleate and arachidonate while FSR contained substantial amounts of eicosapentaenoate and docosahexaenoate. FTIR spectroscopy revealed that the lipids of both membranes did not undergo a phase transition between 0 and 50°C. The lipids were in the liquid-crystalline state at physiological temperatures and underwent monotonic increases in conformational disorder as the temperature was raised. CD spectra indicated higher content of α-helical structure of proteins in RSR than in FSR. Increasing temperature caused diminution of α-helix content. Relatively large decreases in ellipticity were observed between 20°C and 40°C for FSR and 30°C and 60°C for RSR. Measurements of intrinsic tryptophan fluorescence as a function of temperature gave similar results for membrane proteins in both FSR and RSR. The rate of change of tryptophan fluorescence and fluorescence lifetimes was constant over the temperature ranges studied, and no abrupt shifts in fluorescence occurred in the temperature regions where ellipticity decreased rapidly.

Introduction

Many species of lipids exist in any given biological membrane. It is accepted that the physical milieu created by membrane lipids can influence the physiological and biochemical functions of the membrane. The details of lipid–protein interactions, however, are far from being resolved as yet. We do not know, for example, why one membrane may have a substantial quantity of chains with two or four double bonds in its lipids, whereas another might have a high quantity of chains with six double bonds. Physical-chemical studies on model systems suggest that such changes in degree of unsaturation may not cause major changes in bulk properties, such as overall viscosity, in the membrane. The membrane-specific occurrence of selected lipids, however, might well cause more subtle differences between their respective membranes, such as different degrees of mixing or demixing of lipids, or selective interactions at specific protein/lipid interfaces.

In biological membranes, contrary to model systems, the permutation of the various headgroups, chain

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Abbreviations: CD, circular dichroism; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; FSR, flounder sarcoplasmic reticulum; FTIR, Fourier transform infrared spectroscopy; K_m, Michael-Menten constant; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; RSR, rabbit sarcoplasmic reticulum; V_m, maximum initial velocity.

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lengths and degrees of unsaturation results in the existence of thousands of different lipids, of which several hundreds can be present in the same membrane. Yet the lipid/protein ratio is relatively well defined for each membrane (for review see, for example, Ref. 1). This suggests a rigorous adaptation of the lipid composition to specific functions and specific environments. As a part of our on-going studies on the physical-chemical and physiological consequences of lipid variations in biological membranes adapted to different conditions, we have begun a detailed investigation of (Ca^{2+} + Mg^{2+})-ATPase in the sarcoplasmic reticulum of winter flounder which can live over a range of −1.8°C to 25°C, and of rabbit with a body temperature of 37°C. We have observed that the temperature optima, denaturation temperatures, effective ATP binding enthalpies, activation energies and the temperature dependencies of K_m and V_m were different for the enzyme from the two sources [2]. This investigation is aimed at helping to elucidate whether differences in the proteins or the lipids, or both, between the two membrane sources may be associated with the kinetic differences.

Methods

Sarcoplasmic reticulum from white skeletal muscle from the back and legs of New Zealand rabbits (RSR) and the white expaxial muscle of winter flounder (FSR) were prepared as described by East and Lee [3]. After being caught by divers, the flounder had been maintained in tanks of running sea water at environmental temperature for 2–6 weeks before use. Samples of sarcoplasmic reticulum were stored in liquid N_2 in 0.25 M sucrose, 1 M KCl, 50 mM potassium phosphate (pH 8.0) at concentrations of between 23 and 44 mg protein/ml. The materials showed one major band on gel electrophoresis corresponding to a molecular mass of 100–110 kDa, consistent with that of (Ca^{2+} + Mg^{2+})-ATPase. Visual inspections of the gels after staining with Coomassie blue suggested that the band comprised 70% or more of the protein present. The preparations remained active for over six months under these conditions, but the samples examined in these experiments were used after shorter storage periods.

Total lipid extracts were obtained by the method of Bligh and Dyer [4] from suspensions of membrane preparations using solvents containing ≈ 0.01% hydroquinone. Lipid extracts were stored in the dark at −70°C. Manipulations were carried out as much as possible in reduced lighting conditions and in the presence of solvents purged with N_2 or Ar to remove O_2. Phospholipids were separated by thin-layer chromatography of silica gel G according to the method of Touchstone et al. [5] using a solvent mixture of chloroform/ methanol/ 2-propanol/ water/ triethylamine (30:9:25:7:25, v/v). After charring with 70% H_2SO_4, phospholipids were quantitated by phosphorus assay as described previously [6,7].

Following 4.5 hours of transmethylation of the lipid in a mixture of absolute methanol and concentrated sulphuric acid (47:3, v/v) plus a small amount (≈ 0.01%) hydroquinone at 65°C, the methyl esters were extracted [7] and the fatty acid composition was determined by gas chromatography using a Perkin-Elmer 8310 apparatus (Norwalk, CT) equipped with 30 m × 0.25 mm capillary column coated with SP2330 (Supelco Inc., Bellefonte, PA) operated at 180°C. The injection port and detector oven were maintained at 230°C. The carrier gas was helium and flame ionization detection was employed. Methyl heptadecanoate was used as an internal standard. Peaks were identified by comparison of retention times with those of known standards.

Protein content was determined by the method of Lowry et al. [8]. ATP hydrolysis activity of (Ca^{2+}+ Mg^{2+})-ATPase was estimated by incubation of 10 or 25 μg of RSR or FSR membrane protein, respectively, in the presence of 50 mmol l^{-1} 3-morpholinopropanesulfonic acid (pH 7.0), 5 mM $MgCl_2$, 1.1 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} EGTA and 80 mmol l^{-1} KCl. The reaction was started by addition of ATP (final concentration 1 mmol l^{-1}). Hydrolysis of ATP was monitored spectrophotometrically using the coupled assay method of Anderson and Murphy [9]. This method employed 6 units per ml of pyruvate kinase (Sigma), 10 units per ml of lactate dehydrogenase (Sigma), 2 mM phospho-*enol*pyruvate and 0.28 μg NADH in the reaction cuvette.

Samples for Fourier transform infrared spectroscopy (FTIR) were prepared by suspending the materials in 2 mM Tris, 10 mM KCl, 0.1 M sucrose (pH 6.8), and centrifuging them to obtain a pellet which was about 10% solids. The pellets were examined using a Harrick cell (12 μm path length) equipped with CaF_2 windows, a 'Teflon' spacer and a jacket through which thermoregulating fluid was passed. Interferograms were acquired with a Sirius 100 spectrophotometer (Mattson Instruments, Inc., Madison WI) equipped with a HgCdTe detector. Routinely, 200 interferograms were collected, co-added, apodized with a triangular function, subjected to one level of zero filling and Fourier transformed to give a resolution of 4 cm^{-1} with data encoded every 2 cm^{-1}. Temperature was controlled (estimated accuracy, 1°C) with a circulating bath (Model A80; Haake Buchler Instruments, Inc., Saddle Brook, NJ) and monitored with a digital thermometer whose thermocouple sensor was placed close to the windows of the cell but not in the optical path. Frequencies were measured with a parabolic peak picking program supplied with the instrument software. The spectrum of water, matched for temperature and path length, was subtracted from all spectra. Residual sloping base-

lines were removed with a linear baseline routine. Control experiments with sucrose have shown that its contribution to the CH stretching mode is negligible under these conditions.

Circular dichroism (CD) and fluorescence measurements were made in the assay buffer described above (no ATP, no coupling factors present) at a concentration of 0.2 mg/ml for CD and 10 μg/ml for fluorescence measurements. During these measurements temperatures were maintained within 0.5 C° or less of the nominal temperature. The spectra shown are representative of those obtained from at least two samples.

Circular dichroism spectra in a temperature range of 4–80°C were recorded on a JASCO J-500A spectropolarimeter using a jacketed far-UV quartz cell with an effective path length of 0.1 cm. After equilibration for 5 min at each temperature 16 spectra in the range 200–250 nm were collected, the signals were averaged and the corresponding baseline of the buffer was subtracted. Samples were then heated to the next temperature. The total heating equilibration and measurement time was approx. 15 min for each temperature studied. The α-helix content of proteins was calculated from the average spectra according to Siegel et al. [10]. A value of 110 was used for the mean residual weight for amino acids in $(Ca^{2+} + Mg^{2+})$-ATPase from the sarcoplasmic reticulum [11]. The calculations were made for 13 data points at various wavelengths for each spectrum. Data are presented as the estimated value ± 1 S.D. of the estimate.

Measurements of intrinsic tryptophan fluorescence in membrane proteins of SR were obtained by three different methods. All three types of fluorescence measurements were obtained over the range 10–50°C with the absorbance of the sample below 0.15 at 295 nm. The heating equilibrations and measurement times were the same as those used for CD measurements. Absorption spectra were measured on a Varian DMS 200 spectrophotometer.

Steady-state fluorescence was measured on a Shimadzu RL-J40 spectrofluorometer in the ratio mode with polarizers to eliminate anisotropic effects. Corrections were made for the signal from the appropriate blank and the wavelength dependence of the instrument response. The excitation wavelength was 295 nm and the emission spectra were recorded in a range 300–450 nm.

The steady-state fluorescence anisotropy was also measured on SLM 8000 C spectrofluorometer with polarizers. The fluorescence anisotropy, r, was defined as:

$$r = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}} \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities with the analyzer parallel and perpendicular to the vertical polarizer, respectively. G represents the ratio of sensitivities of the detection system for vertically and horizontally polarized light [12].

Time-resolved fluorescence measurements were performed by using the technique of time-correlated single photon counting with instrumentation described in detail previously [13]. The excitation source was a cavity-dumped dye laser synchronously pumped by an actively-locked argon ion laser (Spectra Physics) operating at 825 kHz with a pulse width of 15 ps. Emission at 320, 340, or 360 nm following vertically polarized excitation at 295 nm was detected (right-angle geometry) after passing through a polarizer set at 55° to the vertical and a Jobin Yvon H10 monochromator (Jobin Yvon Instruments SA, Metuchan, NJ), with a 4-nm band-pass, on a Hamamatsu 1564U-01 microchannel plate photomultiplier. Data were collected in 1024 channels with a width of 21.6 or 10.8 ps/channel. A buffer blank was also measured under conditions identical to those of the corresponding sample. The obtained data were analyzed with the non-linear least-squares iterative convolution method based on the Marquardt [14] algorithm. The criteria for the adequacy of a particular experimental model which were applied in these analyses are discussed in Refs. 15 and 16.

Results

$(Ca^{2+} + Mg^{2+})$-ATPase activities of sarcoplasmic reticulum from flounder (FSR) assayed at 29°C (where the enzyme was most active) were 2.9 ± 0.3 ($x \pm$ S.D., $n = 4$) μmol ATP hydrolyzed per min per mg protein. The enzyme from rabbit sarcoplasmic reticulum (RSR) had activities at 37°C between 4 and 7 μmol ATP per min per mg protein.

The phospholipid (taken as 25 times the weight of lipid phosphorus) to protein ratio was independent of season for both SR, and it was slightly higher in FSR (3.0:1) than in RSR (2.2:1). Both membranes had similar contents of phosphatidylcholine (70% of recovered phosphorus) and phosphatidylinositol (6%) as shown in Table I. Phosphatidylethanolamine constituted 15% in RSR and 21% in FSR. Both SR had less

TABLE I

Phospholipid composition of SR (wt% of recovered phosphorus)

	RSR	FSR
Sphingomyelin	3.7	0.7
Phosphatidylcholine	69.8	70.0
Phosphatidylserine	2.5	0.8
Phosphatidylinositol	6.1	5.5
Phosphatidylethanolamine	14.7	20.5
Unknown	3.3	2.6

than 3% phosphatidylserine and less than 4% sphingomyelin.

The fish lipid generally contained more unsaturated chains than that of the rabbit. The compositions were like those found for similar preparations of RSR and FSR previously [2] even though the FSR measured here was obtained from fish at a lower temperature (about −1.5°C) than the FSR measured before [2]. There were significant differences in the nature of the unsaturated chains between the total lipid extracts from FSR and RSR (Table II). As seen previously [2,17], the RSR had high proportions of palmitate (16:0), oleate (18:1), and linoleate (18:2). The FSR had relatively large amounts of the $(n-3)$ polyunsaturated chains, eicosapentaenoate, 20:5 (16.5–18.9%) and docosahexaenoate, 22:6 (23.2%). It contained about 24.5% palmitate, and 9.4–11.8% oleate; there were only small contents of linoleate (0.9–1.3%) and arachidonate, 20:4 (3.0–3.3%). These findings were consistent with observations of Luo and Hultin [18], although these authors found somewhat more $(n-3)$ fatty acids. Fatty acids of the three main individual phospholipids of FSR and RSR (PC, PE, PI), generally reflected the differences in unsaturated chains that were seen in the total lipid extracts. PC, being the most substantial phospholipid, was most similar to the total lipid extract in its fatty acid composition. PE from both sources was the phospholipid which contained the highest amount of unsaturated chains, particularly chains with four, five and six double bonds. PI from both sources showed large amounts of stearate and a fair proportion of arachidonate. It is noteworthy that the high contents of those acids seen in PI from mammalian sources (see, for example, Refs. 19 and 20) occur also in PI from the fish.

The thermotropic behavior of the lipid constituents of RSR and FSR is conveniently monitored [21] through temperature-induced alterations in the frequency of the CH_2 symmetric stretching modes of the lipid acyl chains near 2850 cm^{-1}. This spectral feature is one of the strongest in the IR spectra of phospholipids and suffers no interference from protein bands. The frequency ranges from about 2849 cm^{-1} for gel phase phospholipids to about 2854 cm^{-1} for phospholipids in the liquid crystalline state. Although these frequency changes are small, they may be monitored with a precision of 0.05 cm^{-1} or better. The origin of the frequency increase has been traced by Snyder et al. [22]

TABLE II

Fatty acid composition of lipids of FSR and RSR (wt%)

FSR_1, early summer, water temperatures 8°C. FSR_2, winter, water temperature −1.5°C. n.d., not detected.

Fatty acid	Total lipid				PC		PE		PI	
	FSR_1 [a]	FSR_2	RSR_1 [a]	RSR_2	FSR	RSR	FSR	RSR	FSR	RSR
14:0	3.0	2.4	1.2	1.9	2.9	1.1	1.1	2.4	0.9	1.2
16:0a [b]	0.9	1.1	3.5	5.4	0.9	2.6	1.9	9.3	n.d.	0.5
16:0	24.6	24.1	31.0	25.0	30.9	41.5	10.1	14.5	8.6	11.7
16:1	3.2	1.8	2.2	2.0	3.0	0.7	1.2	0.6	1.0	0.8
18:0	3.5	4.5	7.5	8.0	1.1	2.2	3.1	10.5	27.0	42.8
18:1	11.8	9.4	15.0	19.2	10.5	14.2	14.3	12.2	9.6	5.9
18:2	0.9	1.3	17.1	12.0	1.1	22.2	1.2	7.6	0.8	4.4
18:3	0.2	n.d.	1.4	1.2	0.3	0.9	n.d.	n.d.	0.1	n.d.
18:4	0.1	0.9	n.d.	0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:0	0.2	n.d.	n.d.	0.2	0.2	0.1	0.6	1.8	0.7	n.d.
20:1	3.6	1.6	0.2	0.2	1.7	0.1	9.9	n.d.	5.1	n.d.
20:2	0.2	n.d.	0.3	0.8	0.3	0.4	n.d.	n.d.	1.0	0.7
20:3	0.1	0.2	1.3	1.3	0.2	1.3	n.d.	1.9	0.1	2.4
20:4	3.0	3.3	2.1	8.8	2.3	6.8	3.0	15.1	8.4	16.7
20:5	16.5	18.9	0.8	0.9	18.4	0.7	12.3	1.3	10.4	0.6
22:0	n.d.	n.d.	0.1	n.d.	0.1	n.d.	n.d.	n.d.	0.1	n.d.
22:1	0.4	n.d.	n.d.	n.d.	0.1	n.d.	1.0	n.d.	n.d.	n.d.
22:4	0.2	1.0	1.6	n.d.	0.3	0.8	n.d.	3.4	0.1	n.d.
22:5	2.2	2.4	2.5	3.0	2.3	1.8	3.0	7.6	2.5	2.2
22:6	23.2	23.2	1.0	2.4	22.1	0.5	33.2	2.7	19.5	1.3
24:0	0.1	0.5	0.2	0.3	0.1	0.3	0.5	2.6	0.4	1.8
24:1	0.7	0.8	0.5	2.0	0.8	0.7	1.9	2.4	0.9	1.5
Other	1.4	2.6	4.5	5.0	0.8	1.5	1.8	4.3	0.9	4.6
Total satd.	31.4	31.5	40.0	35.4	35.3	45.2	15.4	31.8	37.7	57.5
	(32.3) [c]	(32.6)	(43.5)	(40.8)	(36.2)	(47.8)	(17.3)	(41.1)	(37.7)	(58.0)

[a] Data from Vrbjar et al. [2].

[b] Tentative identification as hemiacetal of 16:0 aldehyde.

[c] Numbers in brackets include 16:0a.

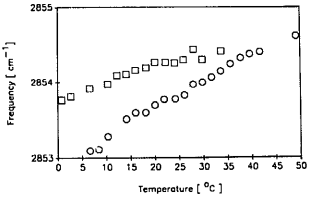


Fig. 1. Temperature dependence of the symmetric CH_2 stretching frequency in the FTIR spectrum of RSR (○——○) and FSR (□——□).

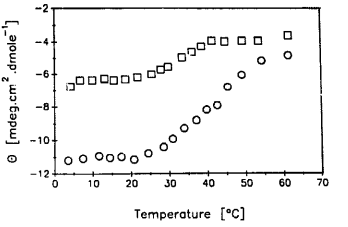


Fig. 3. θ_m (molar ellipticity) at 222 nm for membrane proteins in sarcoplasmic reticulum as a function of temperature. ○——○, Rabbit; □——□, flounder.

to changes in the interaction constants between C–H stretching coordinates on adjacent methylene groups when the lipid physical state is altered (e.g. through the formation of *gauche* rotamers). Although no rigorous correlation exists between the frequency and the extent of acyl chain disorder, the utility of this parameter as a qualitative indicator of the membrane physical state has been amply demonstrated [23].

The temperature dependencies of the 2850 cm^{-1} mode for FSR and RSR are plotted in Fig. 1. Within the limits of the experimental scatter, no indication of a lipid phase transition in either sample is evident over the studied ranges, although a monotonic increase in lipid disorder is seen as evidenced by the frequency increase when the temperature is raised for both RSR and FSR. It is interesting that the frequency for FSR is 0.8 cm^{-1} higher than for RSR at 5°C which is consistent with the substantially higher level of unsaturation in the former leading to more disordered lipid acyl chains. However, the difference in frequency gets smaller as the temperature is raised so that at 30°C, the difference is only 0.2 cm^{-1}.

Fig. 2 demonstrates representative CD spectra of SR membranes at various temperatures. There was less negative elipticity in FSR than in RSR. Elipticity decreased with increasing temperature for both membranes. Plots of measured θ_m at 222 nm (a value often used as an indicator of the α-helix content in proteins) versus temperature suggested a diminution of α-helix content with increasing temperature (Fig. 3). A calculation of the α-helical content from the measured spectra by the method of Siegel et al. [10] as a function of temperature is shown in Fig. 4. Membrane proteins in FSR exhibited lower α-helical content than in RSR. There was a decrease in the calculated α-helical content of the proteins over the range 20–38°C in FSR and between 30 and 60°C in RSR.

The steady-state fluorescence spectra are shown in Fig. 5. In the range 10–50°C the fluorescence of intrinsic tryptophans in both membranes declined progressively with increasing temperature. At all temperatures

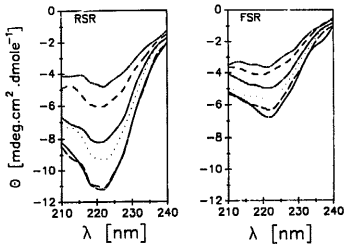


Fig. 2. Representative CD spectra of sarcoplasmic reticulum membranes from rabbit and flounder at various temperatures. RSR: ·-·-·-·-·, 61.4°C; - - - - - -, 49. 1°C; ·-·-·-, 39.7°C; ·····, 33.4°C; ——, 20.7°C; ———, 3.5°C. FSR: ·-·-·-·-·, 61.5°C; - - - - - -, 41.3°C; ·-·-·-, 33.4°C; ·····, 27.8°C; — — —, 18.5°C; ———, 4.2°C.

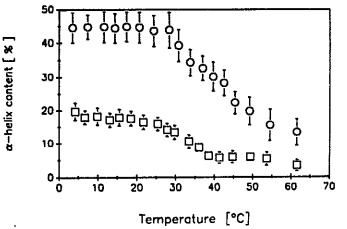


Fig. 4. α-Helix content of membrane proteins in SR as a function of temperature. ○——○, Rabbit; □——□, flounder. The error bars represent ± S.D. of the estimate.

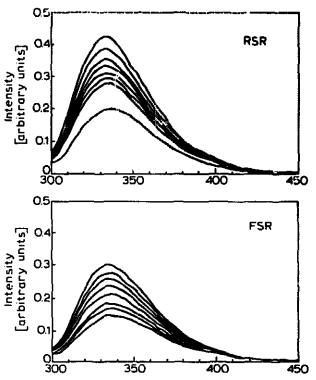


Fig. 5. Steady-state fluorescence spectra of Trp in membrane proteins in SR as a function of temperature. The samples were excited at 295 nm. Fluorescence is normalized to equivalent protein concentrations in both samples. The temperatures of both the samples for the various emission curves were, from greatest to least fluorescence: 10.0, 14.9, 20.0, 23.0, 30.0, 34.8, 39.7 and 49.2°C.

the signal was lower for FSR than RSR. A second set of SR preparations heated from 10 to 65°C showed a similar progressive decline in fluorescence without notable dislocations. Heating caused a progressive red shift (about 5 nm over 10 to 65°C) of the tryptophan emission for both samples. Again, no significant discontinuities with temperature were observed. Plots of the areas under the curves versus temperature resulted in two analogous straight lines (Fig. 6). Fluorescence anisotropy measurements also were similar for both

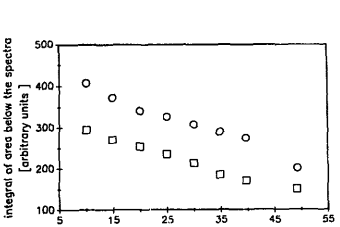


Fig. 6. Intrinsic tryptophan fluorescence in SR as a function of temperature. ○——○, Rabbit; □——□, flounder. Values are normalized per unit of protein in each sample.

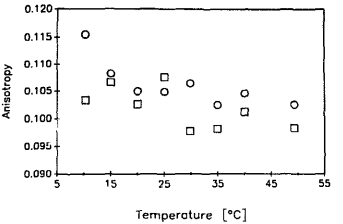


Fig. 7. Fluorescence anisotropy in SR as a function of temperature. ○——○, Rabbit; □——□, flounder.

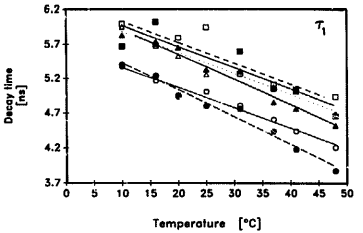
membranes with each showing a slight diminution of anisotropy with increasing temperature (Fig. 7).

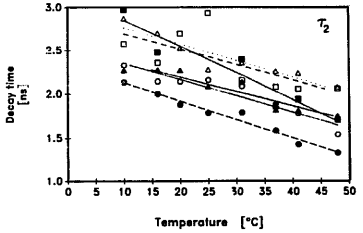
Time-resolved emission measurements were made in an attempt to identify differences in behavior of tryptophan residues in proteins from FSR and RSR. The results of global analysis of decays from a series of emission wavelengths are given in Figs. 8 and 9. A three-exponential fit was required to describe the emission from FSR and RSR at most temperatures. Generally the lifetime of all three components decreased with increasing temperature in similar patterns for both membranes (Fig. 8), although the temperature dependence was very slight for the short lifetime components (Part 3, Fig. 8). Fractions of fluorescence for the two components with shorter lifetimes increased slightly with temperature at the expense of the long-lifetime component (Fig. 9).

Discussion

Our observation showing protein/phospholipid ratios (w/w) of 3:1 in FSR and 2.2:1 in RSR is in agreement with data from other laboratories. Sarcotubular membrane from skeletal muscle of various species exhibit different protein/phospholipid ratios, e.g., rabbit 1.9:1, rat 2.4:1, chicken 3.3:1 and man 4.3:1 [24]. The content of individual phospholipid classes in rabbit SR was similar to results reported by others [24–26]. FSR exhibited a phospholipid composition similar to RSR except it had a slightly higher proportion of PE (20.5%) than RSR (14.7%).

The fatty acid compositions of total lipid extracts of SR from flounder were not substantially different for samples from the fish in the winter (water temperature about −1.5°C) and late spring (water temperature about 8°C). They were characterized by high contents of eicosapentaenoate, and docosahexaenoate, and the predominant saturated fatty acid was palmitate. While these results are generally in agreement with those





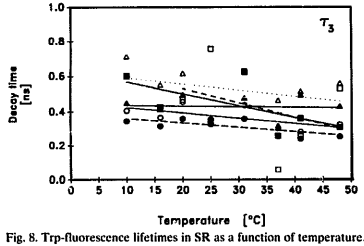
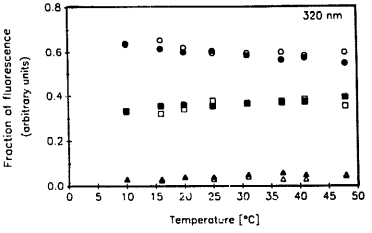
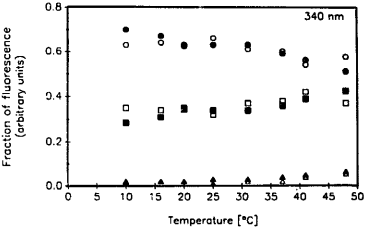


Fig. 8. Trp-fluorescence lifetimes in SR as a function of temperature. Three emission wavelengths were monitored: 320 nm, 340 nm and 360 nm. Open symbols are for RSR and solid symbols for FSR. The three lifetimes are described in separate panels.





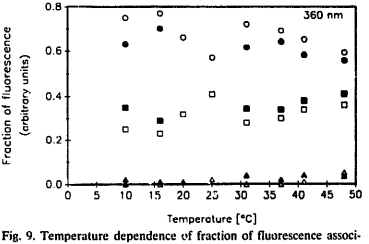


Fig. 9. Temperature dependence of fraction of fluorescence associated with each lifetime: Open symbols are for RSR and solid symbols for FSR. The three emission wavelengths monitored are given in separate panels. Values are normalized per unit of protein in each sample.

published by Luo and Hultin [18], they observed that fish captured in winter had a higher proportion of docosahexaenoate in SR than fish caught in summer. The differences between these observations could have resulted from acclimation of the fish to different local water temperatures. The principal differences between the lipids of FSR and RSR resided in the fatty acids. There was a substantially higher total of 18-carbon (C_{18}) acids (stearate plus oleate plus linoleate) in RSR than in FSR. FSR, on the other hand, had a much

greater proportion of C_{20} plus C_{22} acids than did RSR. Various groups have found a dependence of ATPase activity from RSR on acyl chain length using purified $(Ca^{2+} + Mg^{2+})$-ATPase in reconstituted systems [27–29]. It is possible that differences in chain lengths of surrounding lipid might contribute to higher activity of $(Ca^{2+} + Mg^{2+})$-ATPase in RSR than in FSR at temperatures above about 30°C [2]. However, previous reconstitution experiments of RSR $(Ca^{2+} + Mg^{2+})$-ATPase with species of phosphatidylcholine containing monoenoic chains are somewhat equivocal as to the optimal chain length, with values of C_{18}, C_{20} and C_{22} appearing to be optimal in different studies [27–29].

In contrast to FSR, RSR had high contents of 18:2 and 20:4, typical mammalian $(n-6)$ polyunsaturated acids, and the $(n-3)$ acids were low. Fatty acid compositions of individual phospholipids in RSR and FSR in general reflected the differences as seen in their total lipids. In FSR compared to RSR all major phospholipids exhibited higher proportions of 20:5 and 22:6, together with lower contents of 18:2 and 20:4. These differences might have an influence on the ATPase activities seen in RSR and FSR. Croset et al. [30] found that consumption by mice of diets rich in $(n-3)$ acids resulted in some increase in the proportion of these acids in SR. There were concomitant effects on calcium uptake, but not on ATPase activity of the $(Ca^{2+} + Mg^{2+})$-ATPase. The amounts of the $(n-3)$ acids seen, even in the enriched mammalian membranes, however, were much less than that found in the FSR. Soler et al. [31] found that dietary supplementation with $(n-6)$ acids caused an increase in the $(n-6)$ acid content and a decrease in cholesterol content of chick heart muscle microsomes without having substantial effect on the ATPase or calcium accumulation activities. In this case, however, enrichment was with the $(n-6)$ fatty acids usually found in the microsomes of mammalian muscle, and so the proportions, but not the nature, of unsaturated chains around the ATPase had been changed. The FSR differs from the RSR in both the proportion and the nature of its unsaturated acyl chains.

FTIR indicated that lipids of membranes from both sources are disordered at their respective physiological temperatures. No abrupt change in the amount of lipid motion was seen over the temperature ranges investigated. So there were no direct correlations between changes in the overall lipid order and motion as monitored by FTIR and the abrupt changes in K_m seen for the ATP hydrolytic activity in the two sources [2]. This suggests that any modulation of enzymatic function by the lipid environment is not the result of gross differences in bulk physical properties of the lipids. Based on previous work, gross differences in physical properties of these unsaturated lipids would be unexpected (see, for example, Refs. 32–36). More likely, lipid modulation of enzymatic activity would arise from subtle, but important, differences between the membranes in the ways in which the lipid–protein interactions occur. Two such subtle differences are apparent in Fig. 1. The first is that the degree of disorder and motion indicated by the frequency at their respective physiological temperatures is almost the same for FSR and RSR. Second, the rate of change of the frequency with temperature of the 2850 cm^{-1} mode was less for FSR than for RSR. This indicates that the rate of introduction of disorder into the acyl chains as temperature is increased is less for FSR than for RSR, and that it is controlled by the nature of acyl chain unsaturation. The smaller variability in lipid disorder may be essential for the flounder's viability over the range -1.8°C to about 25°C.

The circular dichroism spectra of the membranes from the two sources provide some insight into the structural changes of proteins in SR induced by variation of temperature. The spectra were checked for the effect of optical flattening by the method of Soos and Fajszi as described previously [37]. In the case of each membrane, the average number of particles in the light path was calculated to be essentially constant over the whole temperature range studied. This suggests that the effect of the vesicular nature of the samples on the CD spectra would be similar at all temperatures. Below 30°C the proteins in RSR exhibit a calculated α-helical content of around 45%, which is in agreement with estimates using other approaches. An α-helix content of 52% in the ATPase has been predicted on the basis of amino acid sequence [38]; Raman spectroscopic studies suggested 50% [39]; FTIR showed 45% [40]; and another CD study [41] yielded 46% of α-helix content for $(Ca^{2+} + Mg^{2+})$-ATPase in rabbit SR. Between 30 and 60°C the α-helical content decreased from 45% to 18%, comparable to the diminution of α-helical content observed by Krebs et al. [42]. The protein is denatured above 55°C as is suggested by other work in this laboratory (unpublished results) which indicates that the enzyme in RSR begins to lose its activity above about 51°C. The activity loss corresponds with thermal denaturation events seen by differential scanning calorimetry [2,43,44]. There was a significant change in the α-helical content of RSR at temperatures (30–45°C) which were below those where calorimetric denaturation and loss of ATPase activity were found. The onset of the change in ellipticity at 222 nm which occurred in the range of 24–28°C corresponded approximately to the temperature where a change in the temperature-dependency of the K_m of the RSR ATPase occurred [2]. The calculated α-helical content of the RSR (Fig. 4) showed a more discrete change between 28° and 32°C which corresponded closely with the change in K_m seen previously [2]. Compared to those in RSR, the proteins in FSR exhib-

ited lower α-helical content at all temperatures studied. There was a diminution in the calculated value of RSR α-helix from 18% at 20°C to a value of 7% at 40°C. As was the case for RSR the temperature where the FSR proteins started to lose α-helical content corresponded approximately, although a little less discretely, to the temperature where the K_m value for $(Ca^{2+} + Mg^{2+})$-ATPase started to rapidly increase [2]. The completion of the change in α-helical content of FSR near 35°C corresponded to the loss in ATPase activity in FSR in a similar fashion to the end of the CD change and activity loss in RSR. A calorimetrically-observable transition was seen in FSR near 35°C which correlated with thermal denaturation of the enzyme [2]. It is perhaps a bit surprising that the two preparations show such a difference in α-helical content. One might expect the structure of the major protein, $(Ca^{2+} + Mg^{2+})$-ATPase, to be similar in both. While we cannot unequivocally eliminate the possibility, we believe that the differences in the CD spectra are probably not due in large part to different degrees of membrane purification [2] or to optical artifacts. The view that some structural differences may exist is strengthened somewhat by the dissimilar behaviour of the RSR and FSR seen in high sensitivity differential scanning calorimetry [2]. Further work on this point, however, would be necessary to reach an unequivocal conclusion.

The CD data suggest that some changes in secondary structure starting at near 20°C for FSR and 30°C for RSR are associated with the changes in the temperature dependencies of K_m for ATP in both FSR and RSR. Total loss of activity is associated with further structural changes at higher temperatures [2]. The reversibility of the CD changes at lower temperature is undetermined. Samples of FSR were heated at 28°C for 2 h in the CD cell and the resulting loss of CD signal intensity relative to that at 15°C was not recovered when the samples were subsequently incubated at 15°C for 20 h. Similarly samples of RSR heated to 37°C for 2 h did not regain signal intensity lost relative to initial values at 20°C when the samples were incubated at 20°C for 18 h or at 20°C followed by 4°C for 18–20 h each. Although these studies suggest that the CD spectral changes may be irreversible, they do not eliminate the possibility of refolding occurring if other protocols were employed. It is noted also that CD signals from the SR do not arise only from the $(Ca^{2+} + Mg^{2+})$-ATPase, although it is the predominant protein of each membrane.

Steady-state fluorescence results showed higher signal intensity for intrinsic tryptophan fluorescence of proteins in RSR than in FSR over the range of temperature studied. Temperature-induced changes in fluorescence spectra were similar for both membranes. The decrease in the fluorescence signals at the wavelength of maximum emission did not display any dramatic changes which corresponded to the changes in CD spectra in the range of 20–40°C for FSR and 30–60°C for RSR. Lepock et al. [44], monitoring emissions at 340 nm in light SR, detected a change in intrinsic fluorescence between 40° and 50°C. On the other hand, East and Lee [3] observed a smooth decrease in intrinsic tryptophan fluorescence from $(Ca^{2+} + Mg^{2+})$-ATPase reconstituted in pure dioleoylphosphatidylcholine vesicles over the temperature range of 10–50°C. The temperature-dependence of tryptophan fluorescence seen here in SR membranes was similar to behavior of tryptophan in glycerol [45]. Thus while the observed decrease in fluorescence intensity could have resulted from structural changes in the proteins, they might also be related to general temperature-induced quenching of fluorescence. In our sample the progression of the emission maximum to higher wavelengths, however, suggests some exposure of the intrinsic tryptophan to a more hydrophilic environment. Thus a structural change that affects the tryptophan environments apparently occurred in our samples, but it was a gradual one over the range of temperature investigated. The emission anisotropy also declined with increasing temperature in a similar fashion for both membranes.

Time-resolved fluorescence spectra suggest that the proteins in both membranes contain at least two populations of tryptophan residues, which are characterized by different fluorescence lifetimes. A third group of tryptophans with the shortest fluorescence lifetime was also observed, but it represented only a low fraction of the fluorescence. The temperature-induced changes of individual fluorescence lifetimes and fractions of fluorescence were similar for RSR and FSR.

The close matching of the fluorescence properties might reflect a similar matching of the structures of the protein and lipid/probe interface and in each membrane. $(Ca^{2+} + Mg^{2+})$-ATPase comprises at least 70% of the SR proteins in both membranes [2]. A three-dimensional model derived from the primary sequence of the ATPase has been proposed, in which 11 out of the 13 tryptophans are located in the intramembranous, hydrophobic portion of the protein [11,38]. Various experiments employing quenching of the membrane-embedded tryptophans have indicated that these fall into two categories, residues that are close to or at the lipid/protein interface, and residues that are more deeply buried in the protein and away from the lipid-protein junctions [3,46,47]. Based on their studies of quenching of tryptophan fluorescence of the $(Ca^{2+} + Mg^{2+})$-ATPase, Froud et al. [46] suggested that about 50% of the intramembrane tryptophan residues are located close to the lipid/water interface. The structural predictions and previous data could support the interpretation that there were tryptophans in three

environments as suggested by our time-resolved fluorescence data.

Our measurements of intrinsic tryptophan fluorescence show no dramatic changes in the temperature ranges studied whereas CD showed loss of structure over a somewhat more defined temperature regions. This might suggest that structural changes observed by circular dichroism are associated with parts of protein which are relatively depleted of tryptophan residues. Thus the changes of secondary structure of proteins of RSR and FSR seen by CD might be related to the extramembranous parts of the $(Ca^{2+} + Mg^{2+})$-ATPase. Since the structural changes observed by CD are associated with changes in ATPase activity [2], these two observations are consistent with the ATP binding site being in the extramembranous portion of the enzyme [44,48,49]. The difficulty with this interpretation is that sequence data would suggest that the membrane-embedded portion of the molecule of RSR, where the major number of tryptophans are located, is also a part of the protein where a great proportion of α-helix may be expected. An alternative explanation is that there are changes in the structure of the transmembraneous portions of the $(Ca^{2+} + Mg^{2+})$-ATPase which are seen by circular dichroism, but not by fluorescence because the tryptophan environments do not change. This might happen if most of the tryptophans in the transmembranous portion of the proteins were exposed to the hydrophobic environment of the lipid chains (see, for example, Refs. 47 and 50), and the nature of their environments did not change dramatically when the unfolding of protein was seen by CD.

The results obtained here suggest that the lipids of FSR may provide an environment a little less susceptible to alterations by environmental temperature than would be that of RSR. This would be advantageous for maintenance of optimal activity of membrane-bound enzymes in the fish. Direct correlations between major alterations in bulk physical properties of the membrane lipids and changes in the kinetic parameters of the enzymes in RSR and FSR were not seen. Structural change in the proteins, seen by a change in CD spectra, began near temperatures where change in the temperature-dependencies of K_m for the $(Ca^{2+} + Mg^{2+})$-ATPase were seen in both SR. While the protein structure changes may be independent of the lipid, further work would be necessary to unequivocally dissociate the structural and kinetic changes from their respective lipid–protein interactions.

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References

1 Devaux, P.F. and Seigneuret, M. (1985) Biochim. Biophys. Acta. 822, 63–125.
2 Vrbjar, N., Simatos, G. and Keough, K.M.W. (1990) Biochim. Biophys. Acta 1030, 94–100.
3 East, J.M. and Lee, A.G. (1982) Biochemistry 21, 4144–4151.
4 Bligh, E.C. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
5 Touchstone, J.C., Chen, J.C. and Beaver, K.M. (1980) Lipids 15, 61–62.
6 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.
7 Keough, K.M.W. and Kariel, N. (1987) Biochim. Biophys. Acta 902, 11–18.
8 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
9 Anderson, K.W. and Murphy, A.J. (1983) J. Biol. Chem. 258, 14276–14278.
10 Siegel, J.B., Steinmetz, W.E. and Long, G.L. (1980) Anal. Biochem. 104, 160–167.
11 MacLen; an, D.H., Brandl, C.J., Korczak, B. and Green, N.M. (1985) Nature 316, 696–700.
12 Lakowicz, J.R. (1983) Principles of Fluorescence Spectroscopy, Plenum Press, New York.
13 Zuker, M., Szabo, A.G. Bramall, L., Krajcarski, D.T. and Selinger, B. (1985) Rev. Sci. Instrum. 56, 14–22.
14 Marquardt, D.W. (1963) J. Soc. Ind. Appl. Math. 11, 431–441.
15 O'Connor, D.V. and Phillips, D. (1984) Time-Correlated Single Photon Counting, Academic Press, London.
16 Drew, J., Zerbetto, F., Szabo, A.G. and Morand, P. (1990) J. Phys. Chem. 94, 4440–4446.
17 Hidalgo, C., Ikemoto, N. and Gergely, J. (1976) J. Biol. Chem. 251, 4224–4232.
18 Luo, S.-W. and Heltin, H.O. (1986) Mech. Ageing Dev. 35, 275–289.
19 Holub, B.J., Kuksis, A. and Thompson, W. (1970) J. Lipid Res. 11, 558–564.
20 Keough, K.M., MacDonald, G. and Thompson, W. (1972) Biochim. Biophys. Acta 270, 337–347.
21 Cameron, D.G., Casal, H.L. and Mantsch, H.H. (1980) Biochemistry 19, 3665–3672.
22 Snyder, R.G., Strauss, H.L. and Elliger, C.A. (1982) J. Phys. Chem. 86, 5145–5150.
23 Mendelsohn, R. and Mantsch, H.H. (1986) in Progress in Protein-Lipid Interactions, Vol.2 (Watts, A.W. and De Pont, J.J.H.H.M., eds.), pp. 103–146, Elsevier, Amsterdam.
24 Marai, L. and Kuksis, A. (1973) Can. J. Biochem. 51, 1365–1379.
25 Meissner, G. and Fleischer, S. (1971) Biochim. Biophys. Acta 241, 356–378.
26 Marai, L. and Kuksis, A. (1973) Can. J. Biochem. 51, 1248–1261.
27 Caffrey, M. and Feigenson, G.W. (1981) Biochemistry 20, 1949–1961.
28 Johannsson, A., Keightey, C.A., Smith, G.A., Richards, C.D., Hesketh, T.R. and Metcalfe, J.C. (1981) J. Biol. Chem. 256, 1643–1650.
29 Froud, R.J., Earl, C.R.A., East, J.M. and Lee, A.G. (1986) Biochim. Biophys. Acta 860, 354–360.
30 Crosei, M., Black, J.M., Swanson, J.E. and Kinsella, J.E. (1989) Lipids 24, 278–285.
31 Soler, F., Alejandre, M.J., Garcia-Gonzales, M., Secovia-Parra, J.L., Fernandez-Belda, F. and Gomez-Fernandez, J.C. (1988) Comp. Biochem. Physiol. 90B, 767–771.

32 Coolbear, K.P., Berde, C.B. and Keough, K.M.W. (1983) Biochemistry 22, 1466–1473.
33 Deese, A.J., Dratz, E.A., Dahlquist, F.W. and Paddy, M.R. (1981) Biochemistry 20, 6420–6427.
34 Stubbs, C.D. and Kouyama, T., Kinosita, K.J. and Ikegami, A. (1981) Biochemistry 20, 4257–4262.
35 Stubbs, C.D. and Smith, A.D. (1984) Biochim. Biophys. Acta 779, 89–137.
36 Keough, K.M.W., Griffin, B. and Kariel, N. (1987) Biochim. Biophys. Acta 902, 1–10.
37 Vrbjar, N., Soos, J. and Ziegelhoffer, A. (1984) Gen. Physiol. Biophys. 3, 317–325.
38 Brandl, C.J., Green, N.M., Korczak, B. and MacLennan, D.H. (1986) Cell 44, 597–607.
39 Williams, R.W., McIntyre, J.D., Gaber, B.P. and Fleischer, S. (1986) J. Biol. Chem. 261, 14520–14524.
40 Villalain, J., Gomez-Fernandez, J.C., Jackson, M. and Chapman, D. (1989) Biochim. Biophys. Acta 978, 305–312.
41 Csermely, P., Katopsis, C., Wallace, B.A. and Martonosi, A. (1987) Biochem. J. 241, 663–669.
42 Krebs, J., Vasak, M., Scarpa, A. and Carafoli, E. (1987) Biochemistry 26, 3921–3926.
43 Gutirrez-Merino, C., Molina, A., Escudero, B., Dietz, A. and Laynez, J. (1989) Biochemistry, 28, 3398–3406.
44 Lepock, J.R., Rodahl, A.M., Zhang, C., Heynen, M.L., Waters, B. and Cheng, K.-H. (1990) Biochemistry 29, 681–689.
45 Demchenko, A.P. (1986) Essays in Biochemistry 22, 120–157.
46 Froud, R.J., East, J.M., Rooney, E.K., and Lee, A.G. (1986) Biochemistry 25, 7535–7544.
47 DeForesta, B., Champeil, P. and LeMaire, M. (1990) Eur. J. Biochem. 194, 383–388.
48 Gutiérrez-Merino, C., Munkonge, F., Mata, A.M., East, J.M., Levison, B.L., Napier, R.M. and Lee, A.G. (1987) Biochim. Biophys. Acta 897, 207–216.
49 Teruel, J.A. and Gomez-Fernandez, J.C. (1986) Biochim. Biophys. Acta 863, 178–184.
50 Lüdi, H., Hasselbach, W. and Gaugler, H. (1985) Biochim. Biophys. Acta 814, 120–124.