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State of aggregation of the $(Ca^{2+} + Mg^{2+})$-ATPase studied using saturation-transfer electron spin resonance

R.M. Napier, J.M. East and A.G. Lee

Department of Biochemistry, University of Southampton, Southampton (U.K.)

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The state of aggregation of the $(Ca^{2+} + Mg^{2+})$-ATPase in the membrane of sarcoplasmic reticulum and in reconstituted membrane systems has been studied using saturation-transfer electron spin resonance (ST-ESR). Saturation-transfer ESR spectra show that in the sarcoplasmic reticulum, the ATPase is relatively free to rotate, with an effective rotational correlation time of approx. 33 μs at 4°C, consistent with a monomeric or dimeric structure. The rate of rotation is observed to decrease with decreasing molar ratio of lipid to protein. In reconstituted systems, rotational motion of the ATPase on the millisecond time scale ceases when the lipids are in the gel phase. Addition of decavanadate, which causes the formation of crystalline arrays in negatively stained electron micrographs, results in only a small reduction in rotation rate for the ATPase in the membrane. The experiments are interpreted in terms of a short-lived (on the millisecond time scale) protein–protein interaction, with the formation of crystalline clusters of ATPase molecules which form and melt rapidly.

Introduction

Proteins are present in biological membranes at very high effective concentrations. Typically, half of the surface area of the membrane might be occupied by protein. It is therefore necessary to consider the nature of the protein–protein interactions in the membrane and the possible importance of such interactions for protein function. Since the protein composition of the sarcoplasmic reticulum of skeletal muscle is unusually simple, with approx. 80% of the protein being the $(Ca^{2+} + Mg^{2+})$-ATPase, the sarcoplasmic reticulum membrane is a very convenient system in which to study protein organisation.

There is much evidence that the $(Ca^{2+} + Mg^{2+})$-ATPase is present in the sarcoplasmic reticulum in oligomeric form. From studies using electron microscopy, it has been concluded that the ATPase is either dimeric [1] or tetrameric [2–4]. From radiation inactivation experiments, Hymel et al. [5] concluded that the ATPase is present as a dimer in the sarcoplasmic reticulum, both in the phosphorylated and in the unphosphorylated forms. Hymel et al. [5] also concluded that the ATPase was dimeric in reconstituted systems containing the purified ATPase at lipid : protein molar ratios of between 52 : 1 and 120 : 1. In a number of studies of negatively-stained samples of

Abbreviations: ATPase, $(Ca^{2+} + Mg^{2+})$-ATPase; ESR, electron spin resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; MSL, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

Correspondence: A.G. Lee, Department of Biochemistry, University of Southampton, Southampton, SO9 3TU, U.K.

sarcoplasmic reticulum using electron microscopy, extensive ordered arrays of the ATPase have been observed. For sarcoplasmic reticulum vesicles purified from rabbit muscle, the ATPase has been observed to form dimers in the presence of decavanadate, the dimers crystallizing to form extensive ladder-like structures [6–8]. In the absence of decavanadate and presence of Ca^{2+}, a crystalline array of rows of monomeric ATPase molecules has been observed [9]. Misra and Malhotra [10] have reported that crystallization of rabbit muscle sarcoplasmic reticulum into rows of dimers can also be induced by treatment with phospholipase A_2. Finally, Castellani et al. [11] have reported that rows of dimeric ATPase molecules occur in scallop sarcoplasmic reticulum, even in the absence of decavanadate.

Electron microscopy gives a 'time-frozen' picture of the membrane. It is, however, also important to know about the lifetimes of any protein–protein interactions, to obtain a picture of the dynamics of the membrane. In a number of studies [12–21] it has been shown that saturation-transfer electron spin resonance spectroscopy (ST-ESR) can be used to study rotational motions of ATPase molecules within the sarcoplasmic reticulum membrane, and that rotational motion is sensitive to the aggregational state of the ATPase. In this paper we will show how saturation-transfer ESR can be used to obtain a picture of the dynamics of protein–protein interactions in the sarcoplasmic reticulum. In the following paper [22], we will show that this picture is consistent with the results of studies of chemical cross-linking of the ATPase.

Materials and Methods

The maleimide spin-label *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide (MSL) was obtained from Aldrich and the phospholipids from Lipid Products.

Electron spin resonance (ESR) spectra were obtained with a Bruker ER 200D spectrometer, interfaced to a Cromemco microcomputer system for spectral accumulation and analysis. All ESR spectra were recorded with a field width of 100 gauss. Conventional ESR spectra (first harmonic absorption in phase, V_1) were recorded with 100-kHz field modulation, with a peak-to-peak amplitude of 2 G and a microwave field amplitude of 0.14 G. Saturation-transfer ESR spectra (ST-ESR, second harmonic absorption out of phase, V_2') were recorded with 50-kHz field modulation, with a modulation amplitude of 5 G and a microwave field amplitude of 0.25 G, essentially as described in Robinson et al. [23]. Saturation-transfer ESR spectra were recorded for samples in a quartz flat cell. The cell was aligned within the magnetic field using markers on the cell and magnet, but spectra were found to be relatively insensitive to the exact orientation of the cell.

Effective rotational correlation times for the ATPase were determined by comparing experimental saturation-transfer ESR spectra with spectra of isotropically tumbling spin-labelled hemoglobin in aqueous glycerol solutions. Spectra for spin-labelled hemoglobin were characterised by ratios of line heights, as shown in Fig. 1, and plotted against rotational correlation times calculated assuming isotropic rotation in a solution whose viscosity was calculated from the data of Slie et al. [24]. The calibration curves obtained in this way agreed well with those published by Kusumi et al. [25] and Thomas et al. [26].

Sarcoplasmic reticulum vesicles and (Ca^{2+} + Mg^{2+})-ATPase were prepared from rabbit muscle (New Zealand White) hind leg muscle as described in East and Lee [27]. Unless otherwise stated, sarcoplasmic reticulum or purified ATPase was labelled with the maleimide spin-label MSL by addition of a concentrated stock solution of MSL in methanol to protein (5–10 mg/ml) in buffer (0.25 M sucrose, 1 M KCl, 50 mM potassium phosphate, 5 mM $MgSO_4$, 5 mM ATP, pH 8.0) to give a molar ratio of MSL : ATPase of 1 : 1. The sample was incubated for 12 h at 4°C then diluted with 8 vols. of buffer (20 mM Hepes, 0.3 M sucrose, pH 7) and spun at $120000 \times g$ for 30 min. The pellet was then resuspended in nitrogen saturated buffer and, working under nitrogen gas, was injected into the ESR flat cell. Although care was taken to maintain samples in a nitrogen atmosphere, Squier and Thomas [33] have shown that the presence of oxygen only decreases the intensity of the saturation-transfer ESR spectrum, with no effects on line shape. In some experiments with sarcoplasmic reticulum, the sarcoplasmic reticu-

lum was prelabelled with *N*-ethylmaleimide for 15 min at 22°C at an *N*-ethylmaleimide : ATPase molar ratio of 1 : 1, before labelling with MSL at a 1 : 1 molar ratio for 4 h at 22°C or 12 h at 0°C.

For lipid depletion experiments, sarcoplasmic reticulum was suspended in buffer (0.25 M sucrose, 1 M KCl, 50 mM potassium phosphate, 5 mM $MgSO_4$, 5 mM ATP, pH 8.0) at 14 mg protein/ml. Potassium cholate was then added to give ratios of cholate to protein (w/w) of up to 2 : 1. The solubilised sarcoplasmic reticulum was layered onto discontinuous sucrose gradients (1 ml 60%, 7 ml 30% and 5 ml 20% (w/v) sucrose) and spun at 95 000 × *g* for 18 h at 4°C. The ATPase formed a band at the 30%/60% interface which was collected and resuspended in buffer. The ATPase was labelled with MSL at a 1 : 1 molar ratio of MSL : protein as described above.

Lipid substitution experiments were carried out essentially as described in East and Lee [27]. Typically ATPase was first labelled with MSL, and the labelled ATPase was then incubated in buffer (0.25 M sucrose, 1 M KCl, 5 mM $MgSO_4$, 5 mM ATP, 50 mM potassium phosphate, pH 8.0) with a 300 : 1 molar ratio of added lipid to ATPase at a cholate : total lipid ratio of 1 : 1 (w/w). For substitution with dimyristoylphosphatidylcholine (DMPC) and dioleoylphosphatidylcholine (DOPC), incubations were 45 min at 22°C and for dipalmitoylphosphatidylcholine (DPPC) were for 15 min at 41°C. Samples were then loaded onto discontinuous sucrose gradients (25%/60%) and centrifuged overnight (95 000 × *g*, 0°C). The lipid substituted ATPase was collected from the 25%/60% interface.

Solutions of decavanadate were prepared by dissolving ammonium vanadate in 0.1 M KOH then bringing the pH down to 2 with HCl and diluting to 50 mM. After a short incubation, the pH was raised again by addition of 120 µl of this 50 mM solution to an equal volume of imidazole buffer (pH 7.4) and adding KOH to pH 7.4 [28]. The decavanadate solutions were used immediately.

Sarcoplasmic reticulum vesicles were incubated in buffer (10 mM imidazole, pH 7.4, 100 mM KCl, 5 mM $MgSO_4$, 0.5 mM EGTA) for 6 h at 4°C with 10 mM decavanadate to induce crystallization, and were then concentrated by centrifugation to approx. 30 mg protein/ml. For electron microscopy, samples were placed on formvar coated grids and stained with 1.5% uranyl acetate for 2 min. Grids were examined in a Hitachi HU-12 electron microscope.

Protein was estimated using the extinction coefficients given by Hardwicke and Green [29] or by the procedure of Lowry et al. [30] using bovine serum albumin as standard. Lipids were estimated by extraction into chloroform/methanol followed by phosphate determination [31]. Lipid : protein ratios were calculated based on a molecular weight of 115 000 for the ATPase.

Results

The conventional ESR (V_1) and saturation-transfer ESR (V_2') spectra of the purified ATPase and sarcoplasmic reticulum labelled with MSL are shown in Fig. 1. The V_1 spectrum of the labelled ATPase shows that the label is strongly immobilized (effective correlation time $> 10^{-8}$). The V_1 spectrum of the labelled sarcoplasmic reticulum is very similar, except for the presence of a small proportion of a weakly immobilized component, as shown by the second peak in the low-field region of the spectrum (Fig. 1). This has been attributed to label bound to a minor glycoprotein component of the sarcoplasmic reticulum containing highly reactive sulphydryl groups [32]. We find that labelling in the presence of ATP at a 1 : 1 molar ratio of MSL to ATPase results in a relatively small contribution from the weakly immobilized component, as shown. Computer subtraction of saturation-transfer ESR spectra with and without the weakly immobilized component were used to obtain the saturation-transfer ESR spectrum of this component at 4°C. The spectrum was found to be similar to that of free MSL in 65% glycerol at 4°C. This component could be computer-subtracted from the saturation-transfer ESR spectra of labeled sarcoplasmic reticulum before determination of the spectral parameters but, in practice, was found to have no significant effect on the measured line height ratios. It has been reported that initial reaction of sarcoplasmic reticulum with *N*-ethylmaleimide reduced the intensity of the weakly immobilized component [20,21]. Under our reaction conditions, however,

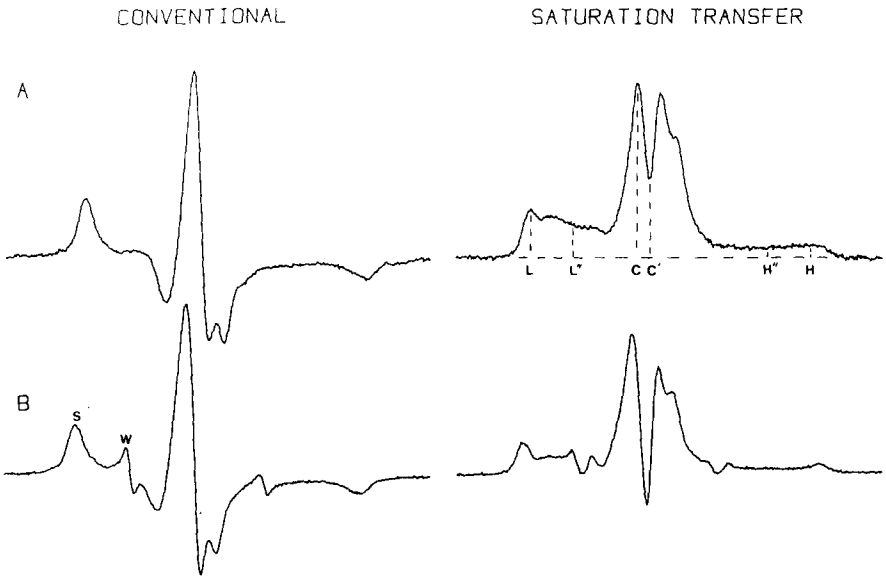

Fig. 1. Conventional (V_1) and ST-ESR (V_2') spectra of (A) ATPase and (B) sarcoplasmic reticulum labelled with maleimide spin label in 0.3 M sucrose, 20 mM Hepes, pH 7.0, 4°C. In the V_1 spectrum, features in the low field region attributed to strongly (s) and weakly (w) immobilised components are marked. In the V_2' spectrum, the positions at which spectral line heights were measured are shown.

we found that prelabelling with *N*-ethylmaleimide had no significant effect on the ESR spectra. Bigelow et al. [20] also reported that heating labelled sarcoplasmic reticulum to 37°C for 3 h reduced the relative intensity of the weakly immobilized component, but we again found that this procedure, whilst leading to a loss of total signal intensity, had little effect on the relative intensities.

The saturation-transfer ESR spectra shown in Fig. 1 are very like those obtained for solutions of hemoglobin labelled with MSL, despite the fact that hemoglobin would be expected to undergo isotropic motion whereas motion of the ATPase in the membrane will be anisotropic. For anisotropic motion, motion cannot be characterised accurately by a single correlation time obtained by comparison with saturation-transfer ESR spectra for an isotropically tumbling species such as hemoglobin. The problem is illustrated in Table I, where the spectra parameters L''/L, C'/C and H''/H are listed for the ATPase together with effective correlation times obtained from calibration curves established with spin-labelled hemoglobin. The very different effective correlation times calculated from the different spectral parameters shows that motion of the MSL label on the ATPase is anisotropic, as expected. Largely because of greater

TABLE I

SATURATION-TRANSFER ESR SPECTRAL PARAMETERS FOR LABELLED ATPase AND DERIVED EFFECTIVE ROTATIONAL CORRELATION TIMES AT 4°C

Molar ratio lipid: protein	Spectral parameter			Effective correlation time (μs)		
	L''/L	C'/C	H''/H	L''/L	C'/C	H''/H
70.9	0.56	−0.32	0.48	33	4	43
21.8	0.87	0.06	0.84	130	14	230
12.5	1.04	0.27	1.0	330	30	500
10.6	1.07	0.30	1.0	600	33	500

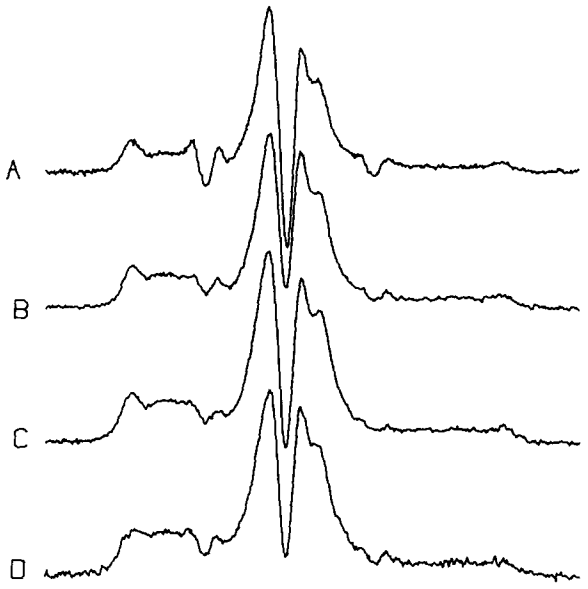

Fig. 2. Saturation-transfer ESR spectra of (A) sarcoplasmic reticulum (lipid:protein molar ratio 89:1) and delipidated samples at lipid:protein molar ratios of: (B), 47:1; (C), 33:1; (D) 20:1. All spectra recorded at 4°C.

experimental difficulty in measuring the ratios C'/C and H''/H, effective correlation times are generally estimated from measurements of L''/L [12–20].

Changing the rate of tumbling of the membrane fragments or vesicles by adding 30% glycerol to the medium to alter the viscosity of the medium, had no effect on the saturation-transfer ESR spectra, demonstrating that the saturation-transfer ESR spectra report on the rate of motion of the ATPase within the membrane, rather than on the rate of tumbling of the membrane itself.

As shown in Fig. 2, saturation-transfer ESR spectra were found to change significantly with changing lipid:protein ratio, consistent with decreasing microsecond protein rotational mobility with decreasing lipid:protein ratio (Table I). Fig. 3 shows how the ratio L''/L changes with changing lipid:protein ratio. The data in Table II show that pH has very little effect on the motional parameters for the ATPase. Addition of 1 mM Ca^{2+} was also observed to have no significant effect on the saturation-transfer ESR spectra.

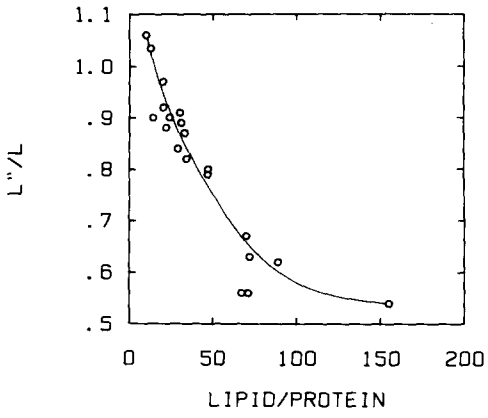

Fig. 3. Plot of the spectral parameter L''/L vs. lipid:protein molar ratio for delipidated sarcoplasmic reticulum samples at 4°C.

The effect of the phase of the membrane phospholipid, liquid crystalline or gel, is illustrated in Fig. 4 and Table III. For lipids in the gel phase, motion is much restricted. In Fig. 5 is shown the saturation-transfer ESR spectrum of a lyophilized sample of the labelled ATPase which is at the slow-motion limit of saturation-transfer ESR. The difference between the spectra is probably due to

TABLE II

EFFECT OF pH ON THE SATURATION-TRANSFER ESR PARAMETERS FOR THE ATPase AT 4°C

pH	L''/L	Effective correlation time (μs)
6.5	0.83	100
7.1	0.77	80
8.1	0.78	84

TABLE III

THE EFFECT OF TEMPERATURE ON THE SATURATION-TRANSFER ESR PARAMETERS FOR THE RECONSTITUTED ATPase

Phospholipid	Molar ratio lipid protein	Parameters, temperature			
		4°C		37°C	
		L''/L	τ_c [a]	L''/L	τ_c [a]
SR [b]	62:1	0.62	41	0.49	25
DOPC	75:1	0.61	40	0.49	25
DMPC	39:1	1.08	400	0.77	80
DPPC	115:1	1.0	250	0.79	85

[a] Effective rotational correlation time (μs).
[b] Delipidated ATPase.

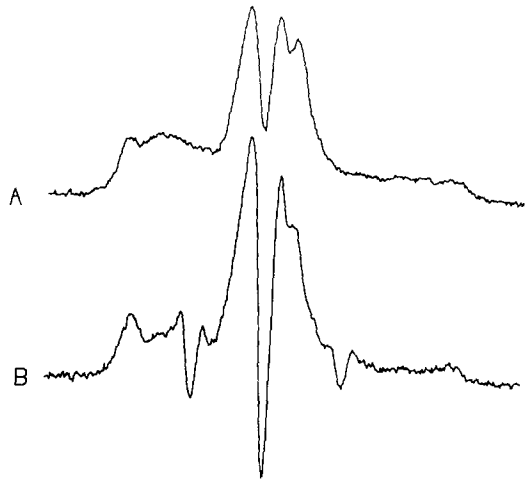

Fig. 4. Saturation-transfer ESR spectra of the ATPase reconstituted with DMPC at a lipid:protein molar ratio of 39:1 at (A) 4° C and (B) 37° C.

motion of the whole membrane. Thomas and Hidalgo [12] have calculated a rotational correlation time of approx. 0.5 ms for tumbling of sarcoplasmic reticulum vesicles in buffer. A similar correlation time for the reconstituted system would give a value for L''/L of 1.1, very close to the observed value for the ATPase in gel phase lipid, implying complete immobilization of the ATPase in the membrane.

The effect of addition of 10 mM decavanadate to the saturation-transfer ESR spectra of labelled sarcoplasmic reticulum is shown in Fig. 5. As shown in Fig. 6, under these conditions electron microscopy of negatively stained samples show extensive crystallization. The value of L''/L for sarcoplasmic reticulum in the presence of decavanadate (0.74 compared to 0.62 in its absence, both at 4° C) implies that the ATPase is relatively immobilized in the presence of decavanadate. The effect of addition of 1 mM Ca^{2+} to sarcoplasmic reticulum vesicles that had been incubated with decavanadate was found to be variable: in some cases, saturation-transfer ESR spectra were found to be unaffected, whereas in other cases the spectra changed to those typical of sarcoplasmic reticulum in the absence of decavanadate.

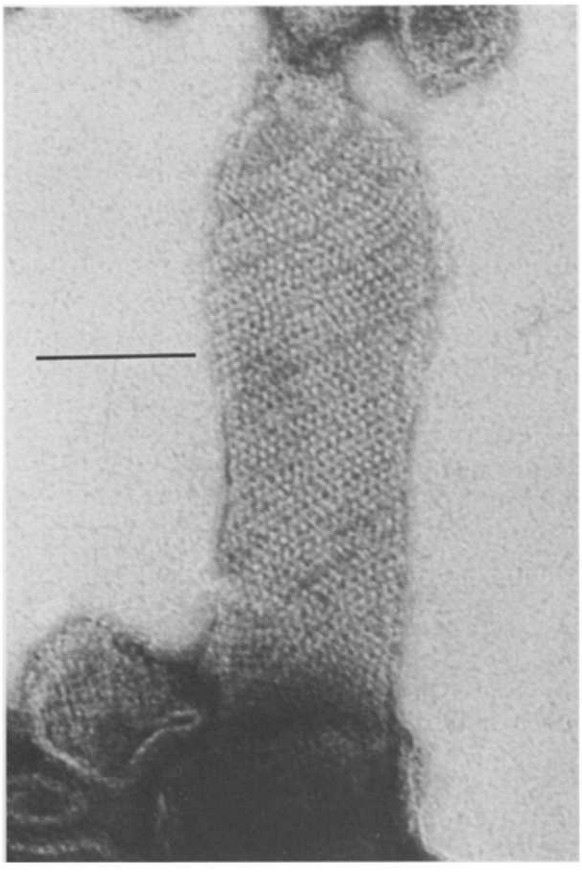

Fig. 6. Sarcoplasmic reticulum vesicles stained with 1.5% uranyl acetate in the presence of decavanadate showing the presence of ordered arrays of protein molecules. The bar represents 0.1 μm.

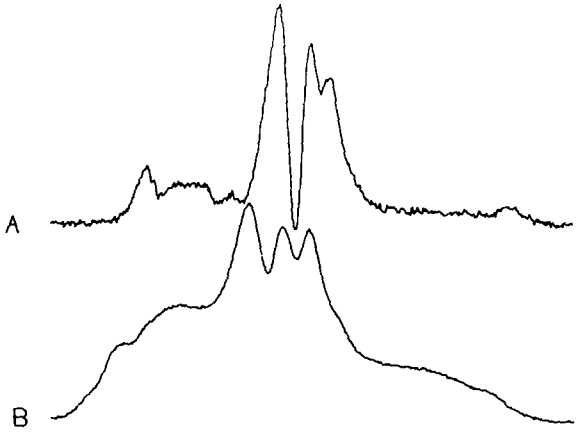

Fig. 5. Saturation-transfer ESR spectra of (A) sarcoplasmic reticulum in the presence of 10 mM decavanadate at 4° C and (B) a lyophilised sample of the ATPase at 18° C.

Discussion

Although motion of ATPase molecules within the sarcoplasmic reticulum membrane would be expected to be highly anisotropic, saturation-transfer ESR spectra for spin-labelled ATPase are very similar to those of isotropically tumbling spin labelled hemoglobin (Fig. 1) as reported by others [12–21]. This could be due to a relatively random labelling of residues on the ATPase by MSL, to some motion of the spin-label group with respect to the whole ATPase molecule, to motion of the labelled domain within the ATPase, or to complex wobbling motions of the whole ATPase molecule. Studies on ATPase mobility using optical spectroscopy have been interpreted in terms of fast internal motions within the ATPase molecule [34,35,42]. Motion of the MSL label is, however, not completely isotropic, since effective correlation times calculated from different features of the saturation-transfer ESR spectra are different (Table I).

Changes in saturation-transfer ESR spectra could be due to changes in the rotational rate of the whole ATPase molecule in the membrane or to changes in the orientation of the spin label with respect to the axes of rotation of the protein. A variety of experiments suggest that the major effects on saturation-transfer ESR spectra follow from changes in the rate of rotation of the ATPase molecule within the membrane [12]. Although it is not possible to obtain accurate correlation times for rotation of the ATPase molecule in the membrane by comparison with saturation-transfer ESR spectra of isotropically tumbling molecules, a number of studies have shown that estimates can be obtained from measurements of the line height ratios L''/L and H''/H (see Fig. 1), and that these can be used to characterise changes in rotational motion of protein molecules [36,37].

Using the equations of Saffman and Delbruck [38] with a membrane viscosity of 2 poise, a membrane thickness of 70 Å and treating the ATPase as a cylinder of diameter either 25 Å for a monomeric species or 45 Å for a dimeric species (see Ref. 9), rotational correlation times of 9 and 25 μs can be calculated for monomeric and dimeric species, respectively. These values can be compared to the value of 33 μs obtained here from measurements of L''/L at high lipid to protein ratios (Table I). The values of L''/L that we obtain are significantly smaller than those obtained for sarcoplasmic reticulum by Lewis and Thomas [21] after pretreatment of labelled sarcoplasmic reticulum by heating to 37 °C.

As shown in Table I, the rotational motion of the ATPase molecule decreases considerably with decreasing lipid : protein ratio. The saturation-transfer ESR spectra obtained at the lowest lipid : protein ratio are near to the slow motion limit of the technique, and, indeed, the correlation time calculated from the spectra is close to that expected for the tumbling of membrane fragments. It is not possible to interpret the spectra obtained at intermediate lipid : protein ratios in detail, because the spectra could either represent a homogeneous population of ATPase molecules rotating at an intermediate rate, or could represent a mixed population of mobile and completely immobile ATPase molecules: spectra such as that shown in Fig. 2 at a lipid : protein molar ratio of 33 : 1, for example, can be reproduced well by an appropriately weighted sum of mobile and immobile saturation-transfer ESR spectra. However, it seems most likely that the changes in saturation-transfer ESR spectra with changing lipid : protein ratio represent at least in part a change in the rate of protein rotation, attributable to a change in membrane viscosity with changing protein content. Such a change in membrane viscosity has been observed for bacteriorhodopsin [39] and an increase in viscosity with increasing protein content would be expected when the protein molecules occupy a significant fraction of the membrane surface area, as they do here.

The results of the lipid substitution experiments shown in Fig. 4 and Table III are in agreement with those of Hidalgo et al. [14]. The effects of temperature on the rotation of ATPase molecules in sarcoplasmic reticulum membranes and reconstituted into DOPC are relatively small, and can presumably be largely attributed to the expected decrease in membrane viscosity with increasing temperature. For the ATPase reconstituted into DMPC and DPPC, however, the effects of temperature are considerably greater. At 37 °C, DMPC will be in the liquid crystalline phase, and the slower observed rate of rotation for the ATPase

observed in this system compared to that reconstituted with DOPC can be largely attributed to the lower lipid : protein molar ratio in the former system (Table III). Although the gel to liquid crystalline phase transition for pure DPPC is 42° C, studies of ATPase activity suggest that the transition is broadened and the temperature of the transition is lowered in the presence of ATPase [27]. The slower rotational rate for the ATPase in DPPC at 37° C than in DOPC (Table III) would then be consistent with a phase separation, with the formation of a bulk phase of DPPC in the gel phase and a phase enriched in ATPase molecules with annular lipid of relatively normal fluidity but decreased protein rotational motion because of the increased local concentration of ATPase molecules. Relative exclusion of ATPase molecules from gel phase lipid has been proposed previously on the basis of studies of lipid binding constants for the ATPase [26] and from freeze fracture electron microscopic studies [40]. For both the DPPC and DMPC systems, protein rotation on the millisecond time scale essentially ceases when the lipids transform into the gel phase at 4° C. This could be due both to a general increase in viscosity for the membrane and to a phase separation of the ATPase molecules into patches highly enriched in ATPase molecules.

Hidalgo et al. [14] reported that addition of high concentrations of Ca^{2+} to sarcoplasmic reticulum vesicles resulted in a reduction in the rate of rotation of the ATPase. We find, however, that addition of Ca^{2+} has no significant effect. The data in Table II show that decreasing pH has a small effect on protein rotation, consistent with a small increase in protein–protein interaction at low pH values.

The results with decavanadate are particularly informative. Electron microscopy of negatively stained sarcoplasmic reticulum has shown that, in the presence of decavanadate, the ATPase crystallizes into ordered arrays of ATPase dimers [6–9]. As reported by Lewis and Thomas [21], we find that crystalline arrays also form for sarcoplasmic reticulum labelled with MSL. Under conditions where electron microscopy shows the formation of crystalline arrays (Fig. 6), saturation-transfer ESR spectra show only a small reduction in rotational rate for the ATPase (Fig. 6), with the calculated effective rotational correlation time increasing from 42 μs in the absence of decavanadate to 70 μs in its presence. Our measured correlation times are significantly shorter than those reported by Lewis and Thomas [21] but the decrease in rotational rate seen on addition of decavanadate is similar. The important result, as noted by Lewis and Thomas [21], is that decavanadate does not cause complete immobilization of the ATPase (compare Fig. 5 with Fig. 4A), as might have been expected from the crystallization shown in Fig. 6.

The most likely explanation for the relatively small effect of decavanadate is that the structure seen in the electron microscope in a time-frozen form is, in fact, dynamic on the millisecond time scale of saturation-transfer ESR. It could be that large scale rotational motion occurs for monomeric or dimeric ATPase species within the crystalline array. Perhaps more likely is a dynamic structure in which linear arrays of ATPase molecules are continually forming from, and breaking up to, individual ATPase monomeric or dimeric species. A relatively short-lived association between any particular ATPase species and the extended array would suggest that only relatively weak interactions were involved in the formation of the array. Rapid collision between ATPase molecules would be expected in the membrane because of the high concentration of ATPase molecules within the sarcoplasmic reticulum membrane. The lateral diffusion rate of the ATPase molecule in the sarcoplasmic reticulum membrane is probably similar to that for rhodopsin in the retinal rod membrane, since Saffman and Delbruck [38] have shown that the rate of diffusion of a membrane protein in a membrane is relatively independent of protein size. Using the lateral diffusion coefficient for rhodopsin measured by Poo and Cone [41], a collision frequency of 10^5–10^6 per second has been calculated between rhodopsin molecules. A very dynamic structure, with rapid (on the millisecond time scale) formation and breaking up of extended linear arrays of ATPase dimers would not then be unexpected. The picture is much like that suggested for the structure of liquid water, with 'flickering clusters' of ordered water molecules present in fully liquid water, in a dynamic equilibrium in which water molecules are free to move from cluster to cluster. The results

described above would then suggest that such structures are longer lived in the presence of decavanadate then in its absence.

In the following paper, these studies will be related to studies of the oligomeric structure of the ATPase using methods of chemical cross-linking.

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