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Effects of Vanadate on the Rotational Dynamics of Spin-Labeled Calcium Adenosinetriphosphatase in Sarcoplasmic Reticulum Membranes[†]

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ABSTRACT: We have studied the effects of vanadate on the rotational motion of the calcium adenosinetriphosphatase (Ca-ATPase) from sarcoplasmic reticulum (SR), using saturation-transfer electron paramagnetic resonance (ST-EPR). Vanadate has been proposed to act as a phosphate analogue and produce a stable intermediate state similar to the phosphoenzyme. This study provides evidence about the physical state of this intermediate. In particular, since ST-EPR provides a sensitive measure of microsecond protein rotational mobility, and hence of protein-protein association, these studies allowed us to ask (a) whether the vanadate-induced protein association observed in electron micrographs of SR vesicles also occurs under physiological (as opposed to fixed, stained, or frozen) conditions and (b) whether vanadate-induced changes in protein association also occur under conditions sufficient for enzyme inhibition but not for the production of large arrays detectable by electron microscopy (EM). At 5 mM decavanadate, a concentration sufficient to crystallize the ATPase on greater than 90% of the membrane surface area in EM, ST-EPR showed substantial immobilization of the spin-labeled protein, indicating protein-protein association in the unstained vesicles. Conventional EPR spectra of lipid probes showed that lipid hydrocarbon chain mobility is unaffected by decavanadate-induced protein crystallization in SR, suggesting that changes in protein-protein contacts do not involve the lipid hydrocarbon region. At 5 mM monovanadate, a concentration sufficient to inhibit the ATPase but not to form crystals detectable by EM, no changes were observed in ST-EPR or conventional EPR spectra of either protein or lipid. In summary, these results indicate that decavanadate induces extensive self-association of the Ca-ATPase but that monovanadate inhibits without a change in protein mobility, thus indicating no change in oligomeric state. If monovanadate produces an analogue of a phosphoenzyme state that is important in the Ca-ATPase cycle, this phosphoenzyme has the same oligomeric state as the unphosphorylated enzyme. Alternatively, if decavanadate produces a phosphoenzyme analogue, the present results suggest that protein association is increased in the phosphoenzyme state.

A central question in studies on the calcium adenosinetriphosphatase (Ca-ATPase) of sarcoplasmic reticulum (SR)¹ concerns the possible oligomeric state of the enzyme. This question is important not only for determination of the enzyme's native state but also for determination of the role protein-protein interactions may play in active calcium transport. Therefore, in order to gain an understanding of the

transport mechanism, it is necessary to know whether the enzyme exists as a monomer or oligomer in its resting state, whether this oligomeric state is important for the cycle, and whether the oligomeric state changes during the cycle.

The Ca-ATPase migrates as a 115-kDa monomer on polyacrylamide gels in the presence of sodium dodecyl sulfate,

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¹ Abbreviations: SR, sarcoplasmic reticulum; EPR, electron paramagnetic resonance; ST-EPR, saturation-transfer EPR; EM, electron microscopy; V_i, vanadate; MSL, *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidyl)maleimide; 5-SASL and 16-SASL, stearic acid lipid probes with the doxyl group at the 5- and 16-positions, respectively; SRB, sarcoplasmic reticulum buffer; CI, crystallization index; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HPLC, high-performance liquid chromatography.

but previous studies have suggested that the ATPase molecule exists as an oligomer in the membrane. The primary evidence for this was obtained from freeze-etch (Deamer & Baskin, 1969; Jilka et al., 1975) and deep-etch/negative-stain electron microscopy (Scales & Inesi, 1976). In the latter study, the particle density on the membrane surface, viewed from outside the vesicle, was found to be 4–6 times greater than that on the intramembrane fracture plane, viewed from inside the vesicle, suggesting that each of the particles in the intramembrane leaflet was an oligomer. It has been proposed that dimers or tetramers of the Ca-ATPase may play a functional role in the transport cycle [for a review, see Martonosi and Beeler (1983)]. A more recent study, using freeze-fracture electron microscopy in conjunction with optical diffraction analysis of the micrographs, has concluded that the observed intramembrane particles represent a dimer of ATPase molecules (Napolitano et al., 1983). Using the technique of radiation inactivation/target size analysis, Hymel et al. (1984) also concluded that the functional unit within the membrane is a dimer.

Studies on the intermediate states of the cycle have provided indirect evidence for an oligomer. In studies of the phosphoenzyme, some reports have concluded that the enzyme can be maximally phosphorylated to only 0.5 mol of phosphate/mol of ATPase [for a review see, Moller et al. (1982)], suggesting either that half of the protein is functionally silent or that the protein is present as a dimer with one phosphorylated site. Using different techniques, other investigators have recently reported a ratio closer to 1 (Barrabin et al., 1984), casting some doubt on this line of evidence for the presence of dimers. However, it has been reported that calcium binding to the Ca-ATPase is cooperative and that this cooperativity can be abolished by the addition of detergents, suggesting protein-protein interactions (Watanabe et al., 1981).

A recent line of evidence involves the phosphate analogue vanadate. Due to its similarity to inorganic phosphate, vanadate has been used extensively in the study of phosphoenzyme intermediates. In the study of the Ca-ATPase, two forms of vanadate have been used, monovanadate and decavanadate (Varga et al., 1985). Decavanadate, the decamer of monovanadate, is formed under low-pH conditions. At or near pH 7, it slowly decays to lower polymeric states. It can be distinguished by its yellow color and absorbance at 400 nm (Maurer & Fleischer, 1984; Varga et al., 1985).

Both forms of vanadate inhibit the Ca-ATPase, presumably by forming an analogue of the phosphorylated state (O'Neal et al., 1979; Dupont & Bennett, 1982; Ortiz et al., 1984; Varga et al., 1985). However, decavanadate has a higher affinity for the phosphate site and may bind to an additional site, resulting in a stoichiometry of 2 mol of decavanadate to 1 mol of Ca-ATPase (Varga et al., 1985). Both vanadate forms have been reported to produce extensive ordered arrays of Ca-ATPase molecules on SR vesicle surfaces (Dux & Martonosi, 1983; Varga et al., 1985), though Maurer and Fleischer (1984) report that only decavanadate is responsible for crystal formation, suggesting that any crystallization by monovanadate must be due to contamination by decavanadate or other vanadate oligomers. This is supported by the observation that decavanadate induces crystallization faster and at lower concentrations than monovanadate. The ordered arrays have been detected in negatively stained, freeze-fracture, freeze-dried, and thin-sectioned electron microscopy (EM) samples (Dux & Martonosi, 1983; Maurer & Fleischer, 1984). By use of optical diffraction, the basic unit within this structure was found to be a dimer (Buhle et al., 1983; Taylor et al., 1984).

In the course of crystallizing, these dimers form ladder-like structures that associate to produce the observed diamond-like pattern. Since the enzyme-vanadate complex may be an analogue of the phosphoenzyme, this suggests that the phosphoenzyme is a dimer. In contrast, crystals formed under conditions designed to mimic the unphosphorylated enzyme contain monomers as the unit structure (Dux et al., 1985). Therefore, the enzyme cycle from an unphosphorylated to a phosphorylated state may involve a monomer-to-dimer transition (Dux et al., 1985).

However, these EM measurements were on a static system and were treated with higher concentrations of vanadate for longer periods than required for enzyme inhibition (Dux & Martonosi, 1983). Thus the following questions must be answered: (a) Does protein association also occur under physiological conditions? (b) Does protein association occur with treatments of vanadate (V_i) sufficient to inhibit the enzyme but not sufficient to cause the extensive arrays needed for detection by EM? Answers to these questions require a spectroscopic probe method that can be applied to native membranes and is sensitive to changes in oligomeric state. Saturation-transfer electron paramagnetic resonance (ST-EPR) measurements of microsecond protein mobility have been shown to satisfy these requirements in SR (Thomas & Hidalgo, 1978; Thomas et al., 1982; Squier & Thomas, 1986b). In the present study, we have spin-labeled the Ca-ATPase and performed ST-EPR measurements of protein mobility, comparing these results with those obtained by EM. In addition, we have used conventional EPR to study the affect of vanadate-induced protein association on lipid hydrocarbon chain mobility.

MATERIALS AND METHODS

Preparation of SR Vesicles. Sarcoplasmic reticulum vesicles were prepared from the white skeletal muscle of New Zealand white rabbits according to the method of Fernandez et al. (1980). The preparation was rapidly frozen and stored in liquid nitrogen until use.

Solutions. All experiments were performed in 0.1 M KCl, 10 mM imidazole, 0.5 mM EGTA, and 5 mM $MgCl_2$, pH 7.4 (Dux & Martonosi, 1983), designated SR buffer (SRB). All vanadate solutions were made from Na_3VO_4 obtained from Fisher Scientific Co. A stock solution of monovanadate was prepared by heating a 0.2 M vanadate solution in an 18 mM glycine buffer at pH 10 until clear, according to the method of Dux and Martonosi (1983). The stock solution was stored in the cold until use. Decavanadate was made by bringing the vanadate solution to pH 2.0 with HCl and then slowly raising it to the working pH of 7.4 with NaOH (Varga et al., 1985). The decavanadate solutions were used immediately. All vanadate solutions were assayed for polymerization by using UV-vis absorption spectra from 230 to 700 nm and comparing them to reference spectra of mono- and decavanadate. In the discussions that follow, vanadate concentrations are given in terms of the total moles of vanadate present without correction for polymerization.

Assays. Protein concentrations were determined by the biuret assay, using bovine serum albumin as a standard (Thomas et al., 1982). The Ca-ATPase content of the total SR protein was assayed by densitometer scans of Coomassie-stained gels of the SR preparation according to the method of Bigelow et al. (1986). The Ca-ATPase content was found to be $78 \pm 5\%$ (by weight). ATPase activities were measured by determining the rate of steady-state phosphate liberation in the presence of 5 mM MgATP; an assay for inorganic phosphate essentially as described by Lanzetta et al. (1977).

was used. The assay procedure was essentially as described by Dux and Martonosi (1983). The SR was incubated in the presence of vanadate and $3.8 \mu\text{M}$ A23187 at 25°C for 15 min prior to the start of the reaction, which was started by the addition of calcium and ATP. The assays were performed at pH 7.4, 25°C , and 0.05 mg/mL protein unless otherwise noted.

Crystallization. Ca-ATPase crystals were produced according to the method of Dux and Martonosi (1983). The crystallization index (CI) is defined as the number of vesicles showing the crystal pattern (as demonstrated by EM) divided by the total number of vesicles, multiplied by 100 (Dux & Martonosi, 1983).

Electron Microscopy. The samples were placed on carbon- and formvar-coated 200-mesh grids obtained from Ernest F. Fullam, Inc. Staining was performed with 1% uranyl acetate. All specimens were prepared at 4°C . A Phillips 201C electron microscope was used at an accelerating voltage of 60 kV. The magnification was calibrated by using carbon replica gratings.

Spin-Labeling. The Ca-ATPase was labeled with a maleimide spin-label, *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidiny)maleimide, designated MSL (obtained from Aldrich Chemicals), according to the procedure of Bigelow et al. (1986). This results in a stoichiometry of 1.2 mol of spin-label/ 10^5 g of SR protein (Bigelow et al., 1986). Analysis of both labeling kinetics (Hidalgo & Thomas, 1977) and the spin distribution on tryptic peptides indicates that the spin-label is attached to more than one site. The stearic acid lipid probes, having the doxyl group at the 5- (5-SASL) or 16-position (16-SASL), were obtained from Aldrich Chemicals and were added to the SR membranes according to the method of Bigelow et al. (1986).

EPR Spectra. Monovanadate EPR samples were prepared by incubation of 30–40 mg/mL protein with 5 mM monovanadate for 1 day at pH 7.4 in SRB. Decavanadate EPR samples were prepared in two ways. In the first case the incubation was performed at 30–40 mg/mL protein concentration in the presence of 5 mM vanadate at pH 7.4 in SRB. In the second case the incubation was performed at 5 mg/mL protein in the presence of 5 mM vanadate at pH 7.4 in SRB, and the membranes were subsequently concentrated inside a dialysis bag by using Aquacide II (obtained from Calbiochem). Both methods yielded identical results.

EPR spectra were obtained with a Varian E-109 EPR spectrometer by following the procedures described in detail by Squier and Thomas (1986a,b). Samples were placed in a capillary made from the gas permeable plastic TPX (Bigelow et al., 1986) and degassed with N_2 for 20 min prior to scanning, since it has previously been shown that O_2 affects both protein and lipid spectra (Squier & Thomas, 1986a). Conventional EPR spectra, which are sensitive to nanosecond motions and are designated V_1 , were obtained with 100-kHz field modulation (with a modulation amplitude of 2 G), using microwave field amplitudes of 0.032 G for the Ca-ATPase spectra and 0.14 G for the lipid spectra. ST-EPR spectra, which are sensitive to microsecond motions, are designated V_2' . Spectra were obtained with 50-kHz field modulation (with a modulation amplitude of 5 G) and a microwave field amplitude of 0.25 G. These parameters were calibrated according to the method of Squier and Thomas (1986a). Spectra were obtained and analyzed according to the method of Squier and Thomas (1986a). In particular, protein rotational motion was characterized by the effective rotational correlation time τ_r , determined by comparing the value of V_2' spectral parameters with calibration plots of these parameters obtained from

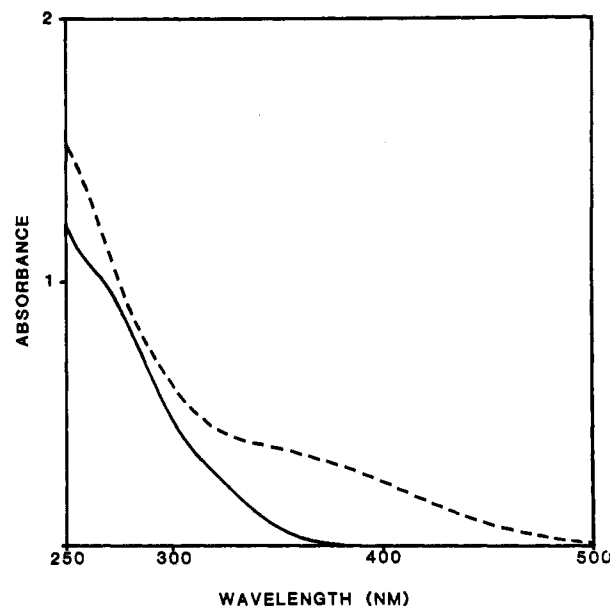


FIGURE 1: UV-vis spectrum of monovanadate and decavanadate solutions at pH 7.4. Solid line, monovanadate; dashed line, decavanadate. The respective vanadate solutions were diluted to a concentration of 0.5 mM in SRB prior to scanning.

spin-labeled hemoglobin in aqueous glycerol solutions (Squier & Thomas, 1986a,b).

RESULTS

Composition of Vanadate Solutions. The UV-vis spectra for monovanadate and decavanadate at pH 7.4 in SRB are shown in Figure 1. On the basis of a calculated extinction coefficient of $550 \text{ cm}^{-1} \text{ M}^{-1}$ under these conditions for decavanadate at 400 nm, the monovanadate contains less than 0.33% decavanadate as a contaminant. However, at this pH monovanadate contains di-, tri-, and tetrapolymers (Chasteen, 1983; Boyd & Kustin, 1984). The decavanadate is stable at 4°C for several days (Varga et al., 1985).

ATPase Activity of Spin-Labeled SR. The spin-labeling procedure changes the Ca-dependent ATPase activity from that of the unlabeled ATPase by less than 5% (Bigelow et al., 1986). The effect of vanadate on the spin-labeled ATPase is virtually identical with that on the unlabeled enzyme. Under standard assay conditions, $50 \mu\text{M}$ monovanadate inhibits the ATPase activity to $19 \pm 3\%$ of the control activity for both the labeled and unlabeled SR. In both samples $50 \mu\text{M}$ decavanadate inhibits the activity to $38 \pm 3\%$ of the control activity. The inhibition by vanadate of the ATPase activity of labeled SR is shown in Figure 2. Fifty percent inhibition is achieved at approximately $0.4 \mu\text{M}$ monovanadate and $3.5 \mu\text{M}$ decavanadate (vanadate concentrations are uncorrected for polymerization). These results on MSL-SR are essentially in agreement with other studies on unlabeled SR (Medda & Hasselbach, 1983; Dux & Martonosi, 1983), but differ slightly with those of Varga et al. (1985), probably due to differences in assay conditions.

In order to determine what effect vanadate has under the conditions used in the EPR experiments, we measured the ATPase activity at high protein concentrations, keeping the ratio of vanadate to SR identical with that in the EPR experiment ($5 \text{ mM } V_i$ and 40 mg/mL protein). For both mono- and decavanadate, the ATPase activity is over 90% inhibited (data not shown). In all cases it was found that the Ca-independent ATPase activity was partially inhibited by vanadate; this was subtracted to obtain the Ca-dependent activity. A concentration of $5 \text{ mM } V_i$ was chosen for all EPR experiments,

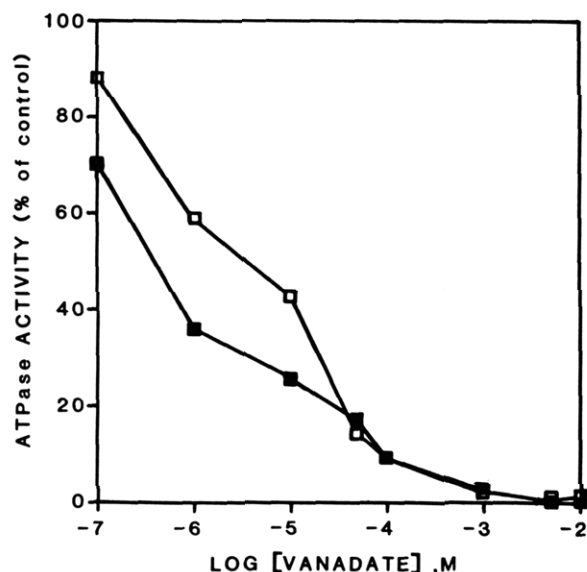


FIGURE 2: Inhibition of spin-labeled calcium ATPase as a function of total vanadate concentration. The Ca-dependent ATPase activity was measured at 25 °C and pH 7.4. The protein concentration was 0.05 mg/mL. Open symbols, decavanadate; closed symbols, monovanadate.

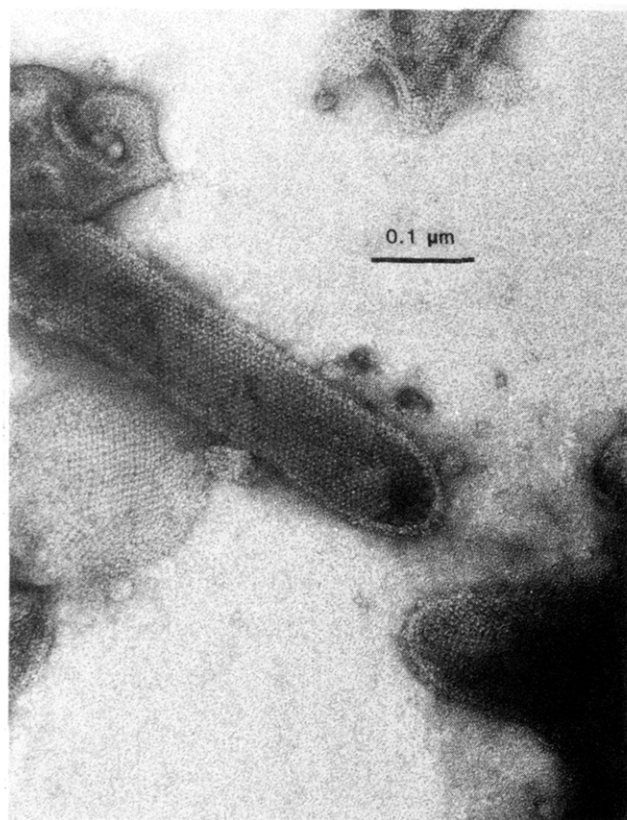


FIGURE 3: SR vesicles stained with 1% uranyl acetate showing the decavanadate-induced ordered arrays ("crystals"). The scale is indicated by the 0.1-μm bar.

since it provided essentially complete inhibition of the ATPase activity and, under the appropriate conditions, complete crystallization.

Electron Microscopy. The data obtained from electron microscopy reveals the characteristic diamond pattern of the vanadate-induced crystal (Figure 3). Under the conditions of 5 mM V_i , pH 7.4 in SRB at 4 °C, crystals formed readily after 1 day of incubation in the decavanadate-treated SR with a crystallization index (CI) of $90 \pm 5\%$. However, in the

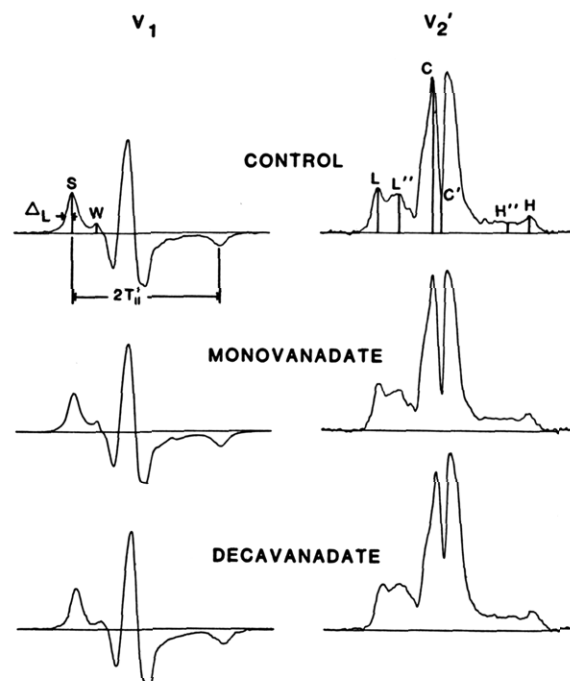


FIGURE 4: EPR spectra of SR vesicles labeled with MSL. The control, monovanadate, and decavanadate spectra are indicated top to bottom. (Left) Conventional (V_1) spectra. Spectral parameters are illustrated on the V_1 spectrum for the control. (Right) Saturation-transfer (V_2') spectra. Spectral line heights are illustrated on the V_2' spectrum for the control. The base line is 120 G wide. Samples were at a protein concentration of 40 mg/mL in SRB at 4 °C.

monovanadate-treated SR the CI was less than 5%. The labeling had no effect on the crystal structure as discernible by EM. The CI was measured both before and after the EPR experiment with the same result. The CI was the same for both conditions used in preparing the decavanadate-treated SR; i.e., it was the same whether the decavanadate incubation was at 40 mg/mL protein or at 5 mg/mL protein with subsequent concentration of the sample.

EPR Spectra. The V_1 EPR spectra for the samples containing MSL-labeled protein (Figure 4, left) have the characteristic line shape of the "rigid limit" for this technique, implying the presence of little or no nanosecond probe motion. More importantly, these spectra show no changes with 5 mM vanadate addition (in the form of either mono- or decavanadate). Thus, although we cannot rule out a small amount of nanosecond motion, we can rule out vanadate-induced changes of nanosecond motions, which would presumably correspond to motions of the probe itself or of a localized region containing the probe. Thus any vanadate-induced changes in V_2' spectra must correspond to changes in microsecond motion that reflect the large-scale rotations of the protein (Thomas & Hildago, 1978; Squier et al., 1985). The V_2' spectra show substantial changes with decavanadate treatment, consistent with decreasing microsecond protein rotational mobility, whereas monovanadate has no detectable effect (Figure 4). A quantitative analysis of these EPR spectra is given in Table I, in terms of the spectral parameters and their corresponding effective rotational correlation times, τ_r . Note that the correlation times determined from different spectral parameters are not identical. These τ_r values are rigorously accurate only if all probes in a sample undergo isotropic motion with the same correlation time. Thus variation in the τ_r values in Table I is presumably due to the parameters' differential sensitivity to anisotropic motion and heterogeneity of correlation times within the sample, including contributions from the weakly

Table I: Spectral Parameters for MSL-Labeled Ca-ATPase^a

Decavanadate, V_1					
	W/S		Δ_L		$2T_{ }'$
control	0.26 ± 0.01		3.02 ± 0.04		68.1 ± 0.1
deca- V_i	0.23 ± 0.03		3.05 ± 0.03		68.1 ± 0.2
deca- V_i /control	0.89 ± 0.08		1.01 ± 0.01		1.00 ± 0.01
Decavanadate, V_2'					
	L''/L	C'/C	H''/H	L''/L_{pp}	$\int V_2'$
control	0.97 ± 0.04 (117 \pm 15)	0.33 ± 0.04 (14.6 \pm 2.2)	0.63 ± 0.04 (58.9 \pm 8.9)	1.34 ± 0.08 (10.1 \pm 1.6)	0.21 ± 0.01 (14.2 \pm 4.4)
deca- V_i	1.04 ± 0.01 (148 \pm 7.0)	0.48 ± 0.03 (24.8 \pm 2.8)	0.75 ± 0.03 (91.0 \pm 10.1)	1.67 ± 0.10 (19.1 \pm 3.6)	0.23 ± 0.01 (23.0 \pm 6.2)
τ_r (deca- V_i)/ τ_r (control)	1.32 ± 0.12	1.78 ± 0.29	1.64 ± 0.28	1.90 ± 0.18	1.67 ± 0.28
Monovanadate, V_1					
	W/S		Δ_L		$2T_{ }'$
control	0.27 ± 0.01		3.02 ± 0.01		67.8 ± 0.1
mono- V_i	0.26 ± 0.01		3.05 ± 0.02		67.8 ± 0.1
mono- V_i /control	0.97 ± 0.01		1.01 ± 0.01		1.00 ± 0.01
Monovanadate, V_2'					
	L''/L	C'/C	H''/H	L''/L_{pp}	$\int V_2'$
control	0.97 ± 0.01 (115 \pm 1.0)	0.37 ± 0.04 (16.7 \pm 2.3)	0.67 ± 0.02 (66.4 \pm 5.3)	1.33 ± 0.02 (9.51 \pm 0.39)	0.22 ± 0.01 (14.9 \pm 3.2)
mono- V_i	0.99 ± 0.01 (123 \pm 4.0)	0.39 ± 0.04 (18.0 \pm 2.4)	0.67 ± 0.03 (67.1 \pm 8.1)	1.37 ± 0.05 (10.3 \pm 1.0)	0.22 ± 0.01 (15.3 \pm 2.6)
τ_r (mono- V_i)/ τ_r (control)	1.06 ± 0.03	1.09 ± 0.13	1.02 ± 0.15	1.08 ± 0.08	1.05 ± 0.05

^a Numbers in parentheses indicate τ_r (μ s). For the V_1 spectra, W/S is the ratio of the low-field peaks, Δ_L is the outer half-width at half-height of the low-field peak, and $2T_{||}'$ is the maximal splitting of the spectrum, as illustrated in Figure 4. Spectral parameters are defined as in Squier and Thomas (1986a). For the V_2' spectra, L'' , L , C' , C , H'' , and H are line-height parameters as illustrated in Figure 4. L_{pp} is the peak-to-trough height for the low-field in-phase V_2' spectral peak at $1/10$ gain. The integral parameter is the intensity of the V_2' spectrum normalized to the number of spins in the sample (i.e., to the double integral of the V_1 spectrum). The ratio is formed from a mean of the ratios from each experiment, and the uncertainty given is the standard error of the mean. Abbreviations: deca- V_i , decavanadate; mono- V_i , monovanadate.

Table II: Spectral Parameters for Lipid Probes in Decavanadate-Treated SR^a

	5-SASL			16-SASL
	$2T_{ }'$	Δ_L	S	τ_r
control	60.5 ± 0.2	3.20 ± 0.05	0.789 ± 0.002	2.83 ± 0.09
deca- V_i	60.3 ± 0.2	3.24 ± 0.05	0.786 ± 0.002	2.89 ± 0.06
deca- V_i /control	1.00 ± 0.01	1.01 ± 0.03	1.00 ± 0.01	1.02 ± 0.01

^a For the 5-SASL spectra, $2T_{||}'$ is the maximal field splitting, Δ_L is the outer half-width at half-height of the low-field peak, and S is the order parameter. For the 16-SASL spectra, τ_r is the effective correlation time in ns.

immobilized component reflected in the W/S parameter in the V_1 spectra (Squier & Thomas, 1986a; Thomas, 1986). This does not seriously affect the interpretation of our spectra, since we are measuring a change from the control value. Decavanadate increased all of the V_2' spectral parameters, with an average increase of $67 \pm 10\%$ (SEM) in the correlation time, whereas the monovanadate spectrum showed no significant change from the control. The corresponding EM data showed crystals only in the decavanadate sample and not in the control or the monovanadate sample.

The conventional EPR spectra for the lipid probes, 5-SASL and 16-SASL, show no changes under the conditions used for the MSL spectra (see Figure 5 and Table II). The sample treated with decavanadate with a CI greater than 90% showed no change from the control spectrum, as revealed by the negligible difference in the spectral parameters (Table II) and the negligible intensity of the difference spectrum (Figure 5). Monovanadate-treated SR also showed no change from the control (data not shown).

DISCUSSION

Summary of Results. Our results indicate that ST-EPR spectra are sensitive to the protein-protein interactions induced

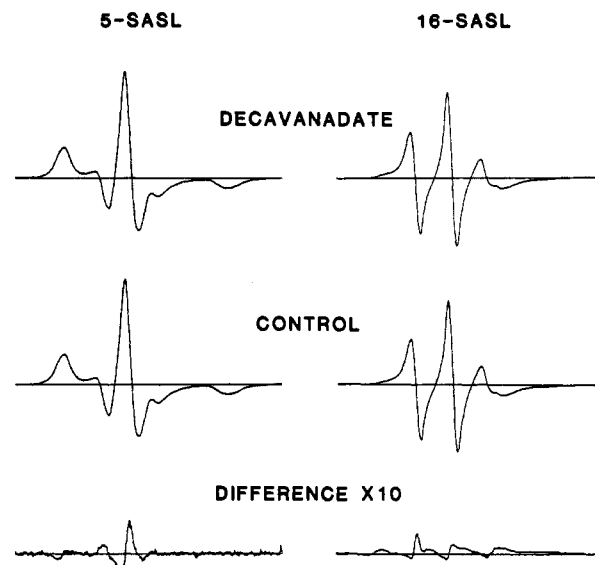


FIGURE 5: EPR spectra of SR vesicles labeled with a lipid probe, 5-SASL (left) or 16-SASL (right). Spectra were recorded in the absence (no V_i) and presence (V_i) of 5 mM vanadate. The difference spectrum, obtained by digital subtraction of the no V_i spectrum from the V_i spectrum, is shown at the bottom. Samples were at a protein concentration of 40 mg/mL in SRB at 4 °C. The base line is 100 G wide.

by decavanadate in SR. Compared to the control sample, the decavanadate-treated Ca-ATPase sample showed substantial changes in the ST-EPR spectrum, corresponding to a $67 \pm 10\%$ increase in the effective correlation time, i.e., a 67% decrease in protein mobility, under conditions where negatively stained membranes have ordered arrays. However, treatment with monovanadate, which inhibits the enzyme but induces no EM-detectable crystals, produced no spectral changes.

Thus there is an apparent direct correlation between the formation of ordered arrays detectable by EM and the decrease in protein mobility as determined by ST-EPR. In addition, the vanadate-induced arrays did not affect the lipid hydrocarbon chain mobility or the lipid distribution between the boundary and bulk lipid populations.

Interpretation. The effect of decavanadate on the mobility of the Ca-ATPase confirms what is suggested by EM, that the motion decreases due to protein-protein interactions. This result is consistent with previous studies in which it was shown that the inhibition of protein mobility correlates with the inhibition of enzymatic activity at the phosphoenzyme decomposition step (Thomas & Hidalgo, 1978; Bigelow et al., 1986; Squier et al., 1985). This suggests a requirement for protein mobility in the Ca-ATPase cycle. However, the results from ST-EPR indicate that decavanadate did not completely immobilize the enzyme on the microsecond time scale, as might have been expected from the electron micrographs. Therefore, protein mobility is still present within the membrane. This mobility can be explained in three ways: (a) There could be intramonomer segmental flexibility, so that even though the monomer is rigidly fixed in the array, motion is occurring in a localized domain within the monomer. Although this possibility cannot be ruled out, previous studies have provided evidence that this spin-label reports the overall rotational mobility of the protein (Thomas & Hidalgo, 1978; Squier et al., 1985). (b) There could be motion of the monomer subunits within an oligomer, corresponding to a wobbling motion. (c) There could be motion of an oligomer relative to the array. In model c, the unstained array would be a dynamic structure, in which the individual oligomers would be allowed to rotate, rather than the fixed structure as seen in electron micrographs. The oligomers that are in this structure have been shown to be dimers (Buhle et al., 1983; Taylor et al., 1984). The correlation times for our decavanadate-treated Ca-ATPase increased by $67 \pm 10\%$, for which the simplest explanation is a substantial increase in the molecular weight of the rotating unit (Thomas, 1985; Squier et al., 1985). If we are detecting a monomer-to-dimer transition, most models for a rigid dimer rotating freely with respect to the array would predict a 2-fold or greater increase in the correlation time. The fact that the correlation time increases by only about 1.7-fold suggests either that there is motion within the dimer (combining models b and c) or that the native state of the enzyme is already partially oligomerized and the increase in the correlation time reflects the maximal amount of additional protein-protein association that can occur. If the enzyme-decavanadate complex is an analogue of a phosphoenzyme intermediate, this result suggests that changes occur in the protein mobility during the transport cycle, possibly corresponding to a monomer-to-dimer transition or some other increase in the oligomeric state.

Although slower motion is the most likely interpretation of the decavanadate-induced increase in effective correlation time, a similar spectral change could also be caused by a reorientation of the probe axis with respect to the plane of the membrane (Polnaszek & Thomas, 1985; Thomas, 1986). While this cannot be ruled out, the multiple binding sites of the probe make this possibility unlikely. In addition, preliminary results on oriented membranes show that there is no single preferred orientation of the probe with respect to the membrane (Thomas Squier, unpublished result).

The results from the studies on the effects of crystallization on the lipid mobility and distribution seem to indicate that the protein-protein association occurs external to the lipid bilayer. This is in accord with the cross-linking studies of Thomas et

al. (1982), in which it was found that while cross-linking decreased the protein rotational mobility and increased the amount of oligomer present, it did not affect either the lipid mobility or distribution. This result would seem to minimize the role for lipids as possible modulators of protein-protein interaction in any transport model.

In contrast to decavanadate, when we studied the effects of inhibitory concentrations of monovanadate on the Ca-ATPase, we found no change in the protein mobility. The most likely interpretation for this is that either (a) no oligomeric changes occur upon the formation of the enzyme-mono-vanadate complex or (b) the oligomer formed is too transient for ST-EPR to measure, i.e., it has a lifetime less than 10 μ s. If the enzyme-mono-vanadate complex is identical with the phosphoenzyme, our data would indicate that there is no change in protein mobility and probably no change in oligomeric state as the enzyme goes from an unphosphorylated to a phosphorylated state. In that case, either the phosphoenzyme is a monomer or the phosphorylated/unphosphorylated enzyme exists as a constant oligomer.

In the interpretation of these results, it is important to know whether the enzyme-vanadate complex is an analogue of the phosphoenzyme intermediate of the transport pathway. The majority of the research has been done on monovanadate. Several workers have shown that monovanadate binds to the same enzyme species as phosphate (O'Neal et al., 1979; Dupont & Bennett, 1982; Ortiz et al., 1984). Decavanadate also appears to bind to this site, although with a higher affinity than monovanadate, and also binds to another site (Varga et al., 1985). Decavanadate is a very effective promoter of crystallization. Although it has been reported that monovanadate can promote crystals under some conditions, under our conditions it does not, even at concentrations where the enzyme's ATPase activity is inhibited greater than 90%. Further work is needed to determine which vanadate complex is the better analogue of the phosphoenzyme.

Relationship to Other Studies of Protein-Protein Interactions in SR. Previous investigations have provided evidence suggesting that the Ca-ATPase exists as an oligomer, even in the unphosphorylated state. Ludi and Hasselbach (1983) observed that detergent decreased the excimer fluorescence between pyrene molecules attached to the Ca-ATPase, suggesting that the Ca-ATPase exists as an oligomer in the absence of detergent, although recent studies have cast doubt on their conclusions due to questions concerning the specificity of labeling (Papp et al., 1985). Vanderkooi et al. (1977) interpreted fluorescence energy transfer between unphosphorylated Ca-ATPase molecules in terms of an oligomeric structure.

Other studies have investigated the effects of phosphorylation and ligand binding on fluorescence energy transfer. Inesi and Watanabe (1982) reported that ATP (under conditions that result in enzyme phosphorylation) had no effect on interprotein energy transfer in the native membrane, thus providing no evidence for a change in protein-protein interactions. However, a decrease in energy transfer was observed when ATP was added to the native membrane in the presence of nonsolubilizing concentrations of detergents, suggesting that phosphorylation by ATP decreases protein-protein interactions in detergent. Yantorno et al. (1983) found that interprotein energy transfer increased upon the removal of detergent and that this restored the Mg sensitivity of phosphoenzyme decomposition.

Other techniques have been used to study the oligomeric state of the enzyme. Andersen and Vilsen (1985), using HPLC

techniques on native SR in the presence of detergent, found that the Ca-ATPase existed in an equilibrium between monomers, dimers, and higher oligomers. ATP, vanadate, and phosphoenzyme turnover promoted a shift toward monomers. Using the technique of radiation inactivation/target size analysis, Hymel et al. (1984) conclude that the Ca-ATPase is a constant dimer unaffected by phosphoenzyme formation, although the size estimate was obtained under conditions that could influence the protein's oligomeric state.

Conclusion. While previous studies suggest that protein-protein interactions occur in SR and are important to the Ca-ATPase mechanism, they provided no conclusive evidence for a change in the oligomeric state in the native membrane. This study shows that decavanadate decreases protein mobility in the SR membrane, probably corresponding to an increase in the size of the oligomeric complex. These results on unstained membranes are consistent with EM data on negatively stained SR showing that decavanadate promotes crystals containing dimers, while crystals formed under non-phosphorylating conditions contain monomers (Dux et al., 1985). Thus, the present study suggests a role for changes in protein-protein association in the Ca-ATPase cycle (Squier et al., 1985; Dux et al., 1985). However, in relating these results to the cycle, it is important to consider whether the enzyme-vanadate complex is the equivalent of the phosphoenzyme found in the pathway. If monovanadate produces a better phosphoenzyme analogue, its lack of an effect on protein mobility would suggest that the oligomeric state is unaffected by phosphoenzyme formation.

Future Work. In the future, this technique should be extended to the effects of other ligands, especially calcium (Dux et al., 1985) and inorganic phosphate, on the Ca-ATPase in order to characterize other intermediates of the cycle. However, while studies like the present one on enzymes trapped in putative intermediates may provide insight, it is essential to extend this study of protein mobility to an actively cycling Ca-ATPase by monitoring directly intermediates such as the phosphoenzyme present during the active transport cycle.

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