Spin-Labelling Studies of Fragmented Sarcoplasmic Reticulum*

Giuseppe Inesi

Mellon Institute, Dept. of Biochemistry, Carnegie-Mellon University,
Pittsburgh, Pennsylvania 15213

William C. Landgraf

Analytical Instrument Division Varian Associates,
Palo Alto, California 94303

Abstract

Vesicular fragments of sarcoplasmic reticulum (SR) were spin labelled with 2,2,6,6-tetramethyl,4-isothiocyanate piperidine-1-oxyl (probe "A") and 2,2,6,6-tetramethyl,4-amino (N-iodoacetamide) piperidine-1-oxyl (probe "B"). Two to five moles of probe "A" or "B" were covalently bound to 10^6 g of membrane protein, with minimal loss of activity (ATPase, Ca^{2+} uptake). The EPR spectra of labelled SR were then studied in various experimental conditions.

Strongly acid or alkaline pH, protein denaturation with urea, and membrane solubilization with deoxycholate produced marked alterations of the EPR spectra of spin-labelled SR, indicating changes in the local environment surrounding the probes, and the occurrence of conformational changes.

A reversible modification of the EPR spectra of probe "A" and an accelerated efflux of accumulated Ca^{2+} were produced by increasing the temperature of SR suspensions from 30° to 40° C. Such a parallel behavior indicates that reversible structural transitions may control membrane permeability and Ca^{2+} efflux.

ATP modifies the EPR spectra of probe "B", suggesting that ATP binding to the membrane induces a structural change involving the local environment of certain sulfhydryl groups. The ATP concentration required for this effect is comparable to that required for activation of ATPase. ADP and ITP are also effective, while pyrophosphate, AMP, and cyclic AMP are not. The effect of ATP is reversible.

In other experiments, 2,2,6,6-tetramethyl piperidine-1-oxyl (probe "C") was equilibrated with concentrated suspensions of SR. The EPR spectra obtained thereafter indicate that probe "C" binds to the membrane fragments, still maintaining a high degree of motional freedom. These spectra were markedly changed by deoxycholate solubilization of the membrane fragments, while they were little affected by protein denaturation with guanidine. These results confirm the hypothesis that the region of distribution of probe "C" into SR, is prevalently constituted by low-viscosity lipids.

Introduction

Vesicular fragments of sarcoplasmic reticulum (SR) have a specific ability for ATP-dependent Ca^{2+} accumulation and exhibit an example of a very active ion pump. ATP hydrolysis, tightly coupled to Ca^{2+} uptake, is also catalyzed by SR[3,6] and a high frequency of active sites on the membrane has been postulated.[7,14]

The possible occurrence of conformational changes in SR has been investigated by optic methods,[14] hydrogen exchange,[4] and fluorescent probes.[17] However, the study of conformational changes in a complex system, such as a suspension of membrane fragments, may require several experimental approaches in order to obtain findings that can be interpreted for their significance to the mechanism of ion transport.

In our laboratory, we have applied to this problem the technique of spin labelling, developed by Professor McConnell.[5,15] Thus, paramagnetic probes (containing a chemically stable nitro-oxyl radical) were covalently bound to reactive groups (—SH, —NH_2) of the (normally non-paramagnetic) membrane, and their EPR spectra were studied under appropriate experimental conditions. In other experiments, membrane fragments were equilibrated with 2,2,6,6,-tetramethyl piperidine-1-oxyl, and the distribution of this probe into the hydrophobic regions of the membrane was detected by studying its EPR spectrum.

Since changes in the rotational freedom of the labels (relative to the macromolecule) are reflected by alteration of their EPR spectra, our experiments produced considerable information about the local environment of the probes, and the effects of some ligands and denaturing procedures.

A preliminary account of this work was previously communicated.[12]

Methods

Fragmented SR was obtained by homogenization and differential centrifugation of rabbit hind legs white muscle.[3] The final suspensions of membrane fragments were further purified by extraction in 0·6 M KCl, centrifugation at 40,000 × g for 40 min and resuspension in 10 mM Tris-maleate (pH 6·8).

The electronmicroscopic homogeneity and the biochemical activities of our SR preparations were previously described.[9]

Protein concentrations were estimated by the Biuret or Folin methods, standardized with micro Kjeldhal determinations.

ATPase activity was determined by following the liberation of inorganic phosphate from ATP, in the presence of SR (40–80 μg protein/ml), 16 mM Tris-maleate (ph 6·8), 80 mM KCl, and various concentrations of $CaCl_2$ and $MgCl_2$. The reaction mixtures were incubated at 25° C for 10 min, and five to eight serial samples were taken. The incubations were interrupted with 5% TCA.

In some experiments ATP hydrolysis was measured in the presence of a phosphoenol-pyruvate–pyruvatekinase ATP regenerating system, and the hydrolytic activity was followed by measuring the formation of pyruvate.[14]

Net Ca^{2+} uptake was measured in the presence of 3 mM $MgCl_2$, 80 mM KCl, 16 mM Tris-maleate (pH 6·8), 0·1 mM EGTA, 0·1 mM $Ca^{45}Cl_2$ (0·03 μC/ml), and 300–350 μg SR protein/ml. Ca^{2+} uptake was initiated by the addition of ATP (3 mM final concentration) to the reaction mixture. At the end of the incubation with ATP, a small volume of the reaction mixture was rapidly filtered (Millipore apparatus, filter No. HA 0·45 μ), and the residual radioactivity in the filtrate was measured with a Packard Tri-Carb liquid scintillation spectrometer. The controls for total Ca^{45} were obtained from samples incubated in the absence of ATP. Therefore, the uptake was strictly ATP dependent.

Spin labelling was obtained by incubating SR with 2,2,6,6-tetramethyl,4-isothiocyanate piperidine-1-oxyl (probe "A"), or 2,2,6,6-tetramethyl,4-amino (N-iodoacetamide) piperidine-1-oxyl (probe "B"). The incubation mixtures contained 0·1–0·2 mM of either probe, 2–4 mg SR protein/ml, and 10 mM Tris-HCl (pH 6·8). After 20 h at 2° C the suspensions were centrifuged, and the excess nonreacted label removed by repeated (5×) centrifugations and washings.

In some experiments concentrated suspensions of SR (25–30 mg protein/ml) were equilibrated with 2,2,6,6-tetramethyl piperidine-1-oxyl (probe "C"), in concentrations between 1×10^{-4} and 1×10^{-5} M. Probe "C" was mixed with SR 10 min prior to EPR measurements.

EPR spectra of the labelled SR were obtained on a Varian E-4 spectrometer. The final samples, examined in a flat quartz cell, contained approximately 7·5 mg protein/ml, 16 mM Tris-maleate (pH 6·8), 80 mM KCl and other additions as required by the experimental schedule.

When indicated, the temperature of the sample was regulated by using a Varian V-4540 temperature controller and a Dewar insert into the cavity. Infusion pumps and a cell equipped with a mixing chamber were used for some determinations.

Results

Satisfactory spin labelling was obtained after incubation of SR with either probe "A" or "B". The labels were strongly bound to SR, and were not removed by repeated washings with 0·05–0·60 M KCl at neutral pH. Extraction of SR lipids with organic solvents (90% acetone, 90% diethylether) did not remove the spin labels, which remained attached to the protein component of the membrane.

It was then assumed that probe "A" reacts with —NH_2, and probe "B" with —SH groups of the protein component of SR. The later assumption was confirmed by the inability to label SR whose —SH groups had been titrated with Salyrgan, prior to incubation with probe "B". Salyrgan is a mercurial reagent, and its use for titration of —SH groups of SR has been previously studied in detail.[7,10]

First moment calculations on the EPR spectra of labelled SR, compared to those of known concentrations of free radical in aqueous solution, indicated the presence of 2–5 moles of either spin label per 10^6 g of protein. This figure corresponds to 2·5–4·0% of the titrable —SH groups in SR.

ATPase and Ca^{2+} uptake activity of SR were not significantly altered by spin labelling in the above-described conditions. This is in agreement with previous studies showing that the activity of SR is only inhibited after blockage of more than 30% of the titrable SH groups (as opposed to 2·5–4·0% blocked by our spin labels).

The EPR spectra of a free "oxyl" radical in aqueous solution and of label "A" and "B" bound to SR are shown in Figs. 1 and 2. The spectra of spin-labelled SR are similar to those of other spin-labelled macromolecules, which show two fairly well resolved components: one manifested by three narrower lines, and arising from a more freely rotating label; the other consisting of three broader asymmetric lines, and arising from labels limited in their rotational freedom with a correlation time of the order of 10^{-8} sec.[2,5,15] These types of spectra have been interpreted as due to different sites of spin

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labelling in the macromolecule, where the motion of the label is restricted to a different degree.[5]

The EPR spectra of labelled SR were not altered by changes in ionic strength, or addition of $MgCl_2$, $CaCl_2$, or chelating agents (ethylenediamine tetra acetate or Ethyleneglycol - bis - [- aminoethyl ether] N,N-tetra acetate).

Deoxycholate—an effective detergent for solubilization of SR—and guanidine, in concentrations used for protein denaturation, produced gross alterations of the EPR spectra of both labels "A" and "B" (Fig. 1 and ref. 12).

Effect of pH

Alteration of the EPR spectra of labelled SR were induced by changes in pH. Since these alterations involved the amplitude, but not the line width, of the components of the spectra, a semiquantitative evaluation was obtained by comparing in each spectrum the amplitude of the two components, and deriving a relative ratio.

Figure 3 shows the relative ratios of the amplitudes of "W" and "T" components, as a function of pH. It is apparent that strongly acid and alkaline pH values increase the amplitude of the weakly immobilized component in the spectra of SR labelled with probe "B". These changes are similar to those observed in erythrocyte membranes, whose —SH groups were labelled with N - (1 - oxyl - 2,2,6,6 - tetra - methyl-1-piperidinyl) maleimide.[2]

The pH effect on the EPR spectra of SR labelled with probe "A" is more complex. A progressive increase in the amplitude of the weakly immobilized component is observed between pH 7 and 9, followed by a rapid reversal and an increase of the "T" component at pH 10.

Effect of Temperature

Temperature variations between 8° and 40° C produce reversible modifications of
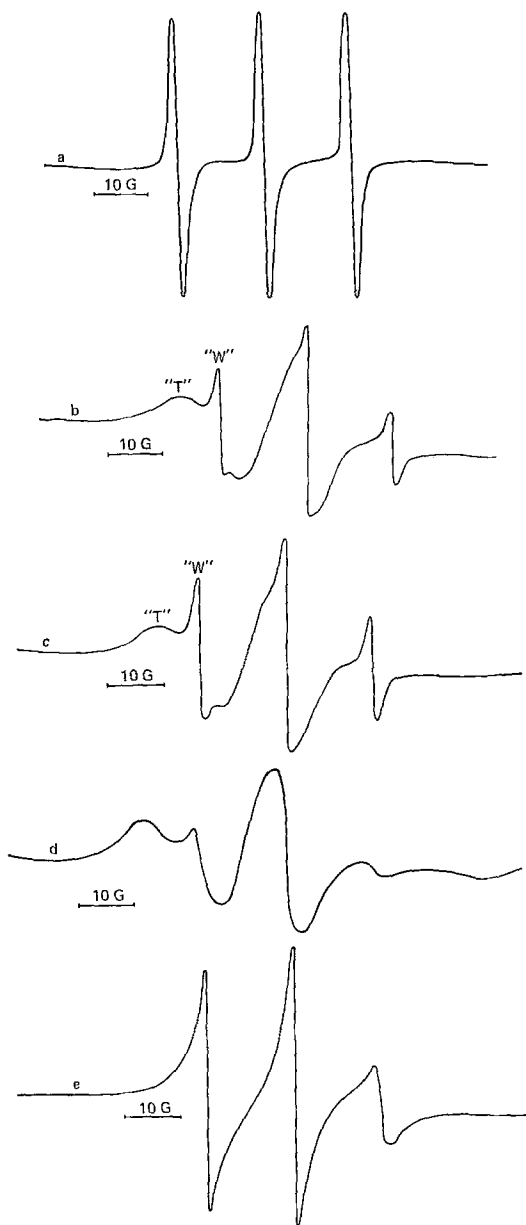

Figure 1. EPR spectra of probe "A" in aqueous solution (a), and bound to SR[(b), (c), (d), and (e)]. Spectrum (b) : sample containing 7·2 mg SR protein/ml, 16 mM Tris-maleate (pH 6·8) and 80 mM KCl; spectrum measured at 25° C. Spectrum (c) : same sample as in (b), but measured at 35° C. Spectrum (d) : as in (b), with the addition of 20 mM deoxycholate. Spectrum (e) : as in (b), with the addition of 6 M guanidine. The EPR spectra were obtained with 1·0 G Mod. Ampl., 70-mW power, 1-sec time constant, 100 G/8 min. field scan rate.

the spectra of SR labelled with probe "A". The effect is most marked between 25° and 38° C, and consists of an increase in the relative "W"/"T" ratios, indicating an increased motional freedom of the label (Fig. 4). No linewidth modification was noticed.

Within the same range of temperatures, no significant alteration was produced on the spectra of spin label "B", which acquired a greater motional freedom only at temperatures higher than 38° C.

A comparison of temperature effect on the EPR spectra of the spin labels and on the functional properties of SR (Ca^{2+} accumulation and ATPase), yields interesting results.

In a previous study on the temperature-dependence of Ca^{2+} uptake and ATPase of SR,[11] identical activation energies were observed for the initial rates of these two activities. Highest steady-state levels for net Ca^{2+} accumulation (in the absence of oxalate) are obtained at 25° C, after 60 sec incubations [Fig. 5(a)]. It was later found that these steady-state levels are reduced when the incubations are carried out at temperatures higher then 25° C [Fig. 5(b)]. The reduction of net Ca^{2+} accumulation is due to changes in membrane permeability, with consequent leak of accumulated Ca^{2+}. Figure 5(c) shows the rates of release of accumulated Ca^{2+} rapidly increasing at temperature above 25° C. The decreased efficiency of the Ca^{2+} pump is also demonstrated by the fact that Ca^{2+}-dependent ATPase, which is normally tightly coupled to Ca^{2+} uptake, is still active within this range of temperatures. ATPase is denatured at temperatures higher than 40° C [Fig. 5(d)], with a diphasic effect of activation and inactivation.

Figure 2. EPR spectra of probe "B" bound to SR. Spectrum (a): sample containing 7·3 mg SR protein/ml, 16 mM Tris-maleate (pH 6·8) and 80 mM KCl. Spectrum (b): as in (a), with the addition of 5 mM ATP. Spectrum (c): as in (a), but measured at 50° C, instead of 25° C. Measurements carried out as described in Fig. 1.

The denaturation produced by temperatures between 30° and 40° C is reversible, inasmuch as good efficiency of the Ca^{2+} pump is obtained when SR is exposed to the experimental temperature prior to the incubation for Ca^{2+} uptake, which is subsequently carried out at 25° C [Fig. 5(b)]. It is interesting to notice the parallel occurrence of reversible modifications in the EPR spectra of label "A" and in membrane permeability, when SR is exposed to temperatures between 30° and 40° C.
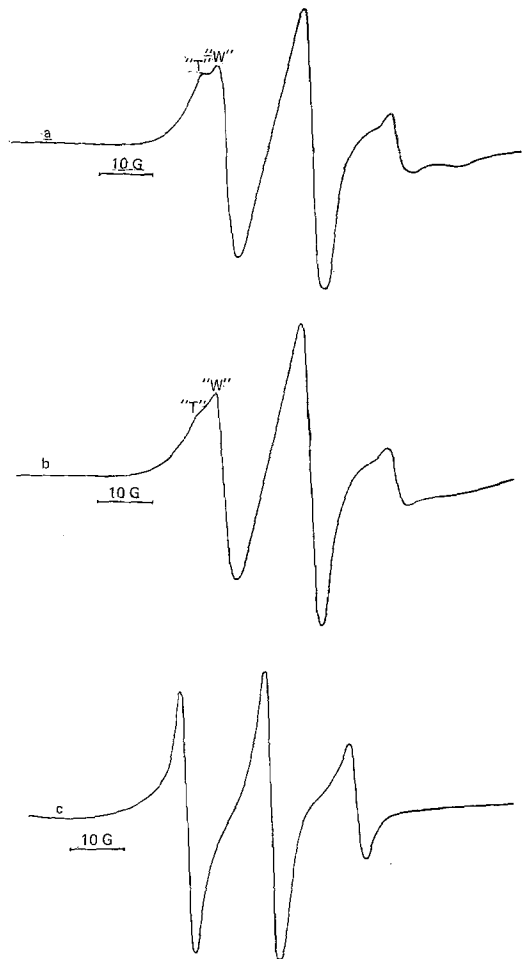
Effect of ATP

Addition of ATP to suspensions of SR labelled with probe "B", increases the relative ratios between the amplitudes of the "W" and "T" components of the EPR spectra (Fig. 2). No individual linewidth modification is associated with these amplitude changes. In continuous-flow experiments it was shown that the effect of ATP is produced within 1 sec after mixing.

Neither Ca^{2+} nor Mg^{2+} was required for the effect of ATP. In the presence of Mg^{2+} and Ca^{2+}, however, the modifications of the spectra slowly reversed, due to dephosphorylation of the nucleotide (Fig. 6). Slower reversal was observed when the ATP concentration was higher.

Figure 7 shows a plot of the relative ratios between the amplitudes of the "W" and "T" components of the EPR spectra of label "B" as a function of ATP concentration. In the same graph, the ATPase dependence on substrate concentration is also displayed for comparison. The later exhibits a two-step sygmoid curve,[10,19] where the apparent Michelis constants are $2 \cdot 5 \times 10^{-6}$ and 3×10^{-4}. The concentration-dependence of the ATP effect on spin-labelled SR is comparable to the second step of the curve of ATPase dependence on substrate concentration.

ITP and ADP, respectively a substrate and an inhibitor of ATPase, also produce an effect similar to that of ATP. Pyrophosphate, AMP, and cyclic AMP are not significantly effective. These results are in agreement with the work of Hasselbach and Seraydarian,[7] who found that ATP and ADP protect SR from —SH reagents, while pyrophosphate, AMP and cyclic AMP do not.

None of the studied nucleotides modified the EPR spectra of SR labelled with probe "A", indicating that the ATP effect on the EPR spectra of label "B" may be due to a localized modification of the membrane structure.
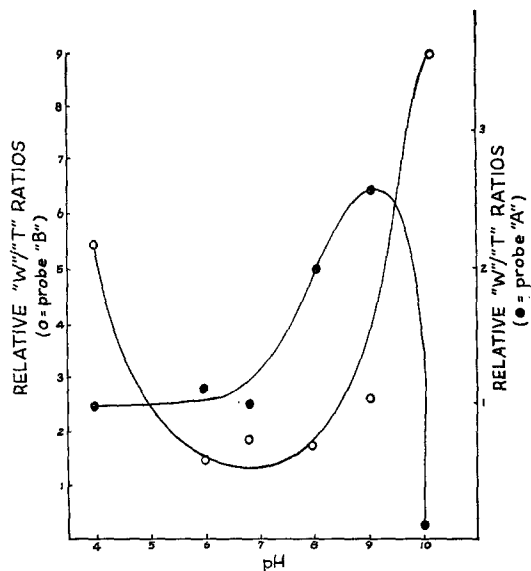

Figure 3. Effect of pH on the relative ratios between "W" and "T" amplitudes in the EPR spectra of SR labelled with probe "A" (●) or "B" (○). Spectra measured at 25° C, as indicated for (b) in Fig. 1.

Distribution of 2,2,6,6-Tetramethyl piperidine-1-oxyl (Probe "C")

In some experiments, concentrated suspensions of SR were mixed with various concentrations of probe "C", and EPR spectra obtained after brief (10 min) equilibration. The EPR spectra of the label in the presence of SR (Fig. 8) appear to be the resultant of two components which are partially resolved in the high field of the nitrogen hyperfine line. These spectra are analogous to those obtained by Hubbell and McConnell, who attributed one of the components to the spectrum of a free label in aqueous phase,

and the other component to the EPR spectrum of the label rotating in a low-viscosity hydrophobic region, with a correlation time of 10^{-9}–10^{-11} sec.[8]

In agreement with this interpretation, we found that when the hydrophobic region of the membrane was disrupted with deoxycholate, the resolution between the two components of the EPR spectrum was obscured. In this condition, the EPR spectrum becomes indistinguishable from that of spin labels in pure detergent solutions.[18]

On the contrary, protein denaturation with guanidine did not change the observed resolution of the two components of the spectrum, while it produced gross changes on the spectra of spin labels "A" and "B", which are bound to the protein component of the membrane (Fig. 1).

Addition of ATP, $MgCl_2$, and/or $CaCl_2$ did not produce observable changes of the spectra of label "C".

Discussion

A point of general interest observed in our experiments is that spin labelling can be usefully applied to the study of membrane systems such as SR. This technique offers the potential of probing selective sites of membranes, where localized conformational changes may be observed. In addition, it overcomes limitations presented to optical methods by the turbity of membrane suspensions.

Spin labelling, to the extent used in our experiments, does not decrease the activity (Ca^{2+} uptake, ATPase) of the membrane, although the possibility should be considered that few sites may be inactivated by the labels, without appreciably decreasing the bulk activity of the preparation.
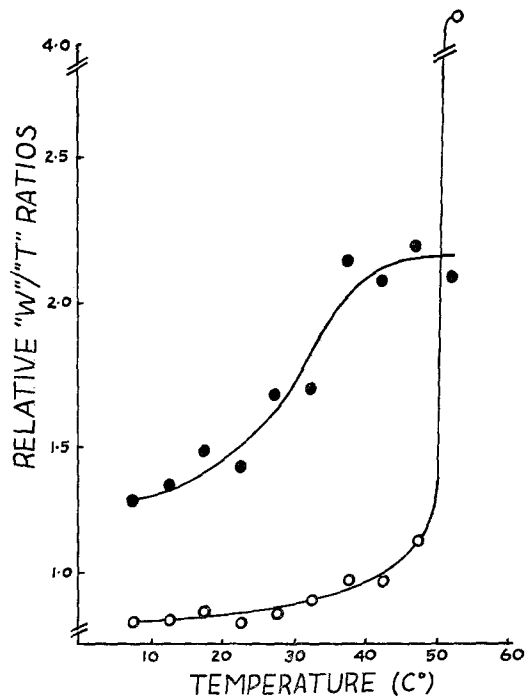

Figure 4. Effect of temperature on the relative ratios between "W" and "T" amplitudes in the EPR spectra of SR labelled with probe "A" (●) or "B" (○). Spectra measured as described for (b) in Fig. 1, but at variable temperature.
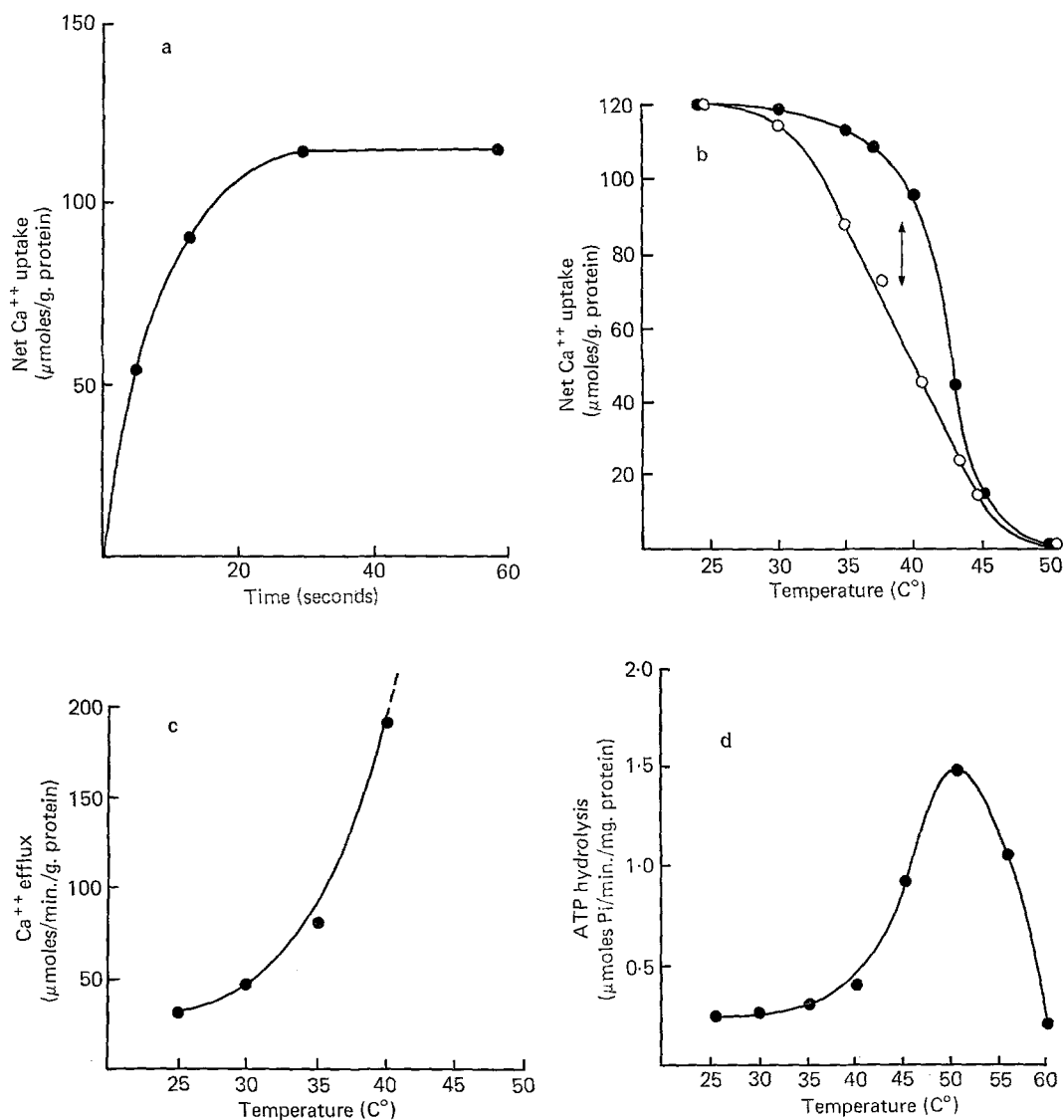
Membrane Permeability and Structural Changes

A variety of experimental procedures has been reported in the literature, which increase SR permeability to Ca^{2+}, thereby producing leak of accumulated Ca^{2+} and apparent uncoupling of Ca^{2+} uptake and ATPase. These procedures, however, involve chemical degradation of the membrane and require the addition of organic solvents,[10] detergents[3] or lytic enzymes.[9] Such modifications are very useful for the understanding of some biochemical properties of SR, and facilitate, among others, kinetic studies of Ca^{2+}-dependent ATPase. It is unlikely, however, that these degradations may represent the mechanism by which rapid structural transitions are produced in SR, to allow Ca^{2+} release in muscle.

On the other hand, stepwise heat denaturation offers a convenient physical means for differential alteration of membrane permeability and ATPase (Fig. 5). Most interesting

Figure 5. (a) Time curve of Ca^{2+} accumulation by SR. Incubation at 25° C, as described in the section on methods. (b) Maximal Ca^{2+} accumulation (60 sec) by SR. Incubations were carried out at the indicated temperature ($\circ$), or at 25° C after 60 sec preincubation at the indicated temperature ($\bullet$). (c) Initial rates of Ca^{2+} efflux from loaded vesicles. SR (300 μg/ml) was incubated in the presence of 3 mM $MgCl_2$, 3 mM ATP, 16 mM Tris-maleate (pH 6·8), 80 mM KCl, 0·1 mM EGTA and 0·1 mM^{45} $CaCl_2$. After 60 sec, Ca^{2+} uptake was interrupted by the addition of 10 mM EDTA (Mg^{2+} chelation), and the samples transferred to a bath regulated at the experimental temperature. Efflux of accumulated Ca^{2+} was determined by filtering samples at different times. (d) Ca^{2+}-dependent ATP hydrolysis, as a function of temperature of incubation.

is the reversible permeabilty change obtained between 25° and 38° C, and the parallel increase in mobility exhibited by label "A". It is likely that the permeability changes are produced by thermal effects on protein unfolding. The consequent structural alteration, revealed by the increased motional freedom of spin label "A", appears to be

reversible, localized (spin label "B" is not affected) and occurs at temperatures of physiological interest.

Denaturation by pH, Urea or Deoxycholate

Strong acid and basic pH produce changes of the EPR spectra of spin label "B" (Fig. 3), indicating a greater motional freedom of the label. This effect is likely related to ionization of some membrane groups which develop repulsive charges (electrostatic) of equal sign. The subsequent expansion of the site would allow a greater rotational freedom for a spin label in that local environment. In addition, the effect of pH on the carboxyl hydrogen bonds of the protein may induce other changes in the membrane structure.

Spin label "A" is affected differently by pH, as compared to label "B". This difference can be understood if one considers that the modifications of the EPR spectra are due to the influence of the local environment on the spin label, not necessarily associated with a general involvement of the macromolecule.

The rapid change of the "W"/"T" ratios of label "B" at extreme alkaline pH is most interesting (Fig. 3). This effect may be explained by the occurrence of new bonds, formed during the spacial rearrangement of the protein.

Deoxycholate is an effective detergent for solubilization of SR.[13] We found that in its presence the EPR spectra of all the three spin labels used in our experiments were grossly altered (Figs. 1 and 8). This observation, which now appears obvious, acquires a greater importance when compared to previous circular dichroism measurements, which did not reveal any modification after solubilization of SR with deoxycholate.[14] The spin-labelling experiments certainly provide strong evidence for the occurrence of conformational changes on membrane solubilization.

Several mechanisms can be proposed for the conformational effect of deoxycholate: the detergent may simply interfere with
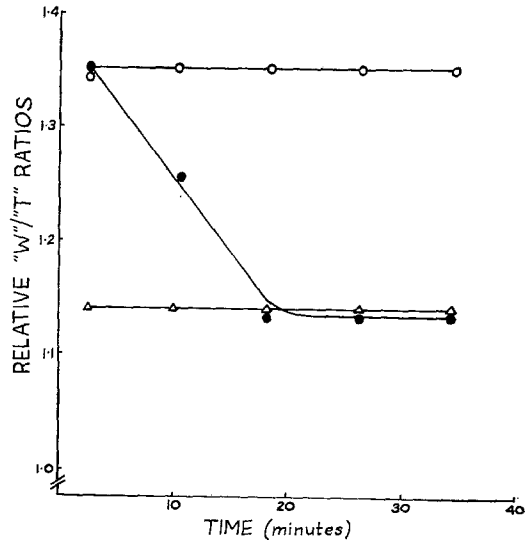

Figure 6. Relative ratios between "W" and "T" amplitudes in EPR spectra of SR labelled with probe "B". (△): 6·9 mg membrane/ml, 16 mM Tris-maleate (pH 6·8), and 80 mM KCl. (○): as △, with the addition of 1 mM ATP. (●) as in △, with the addition of 1 mM ATP, 1 mM $MgCl_2$ and 0·1 mM $CaCl_2$. EPR scans were initiated 2 min after the addition of ATP, and repeated every 8 min. Temp.: 25° C.
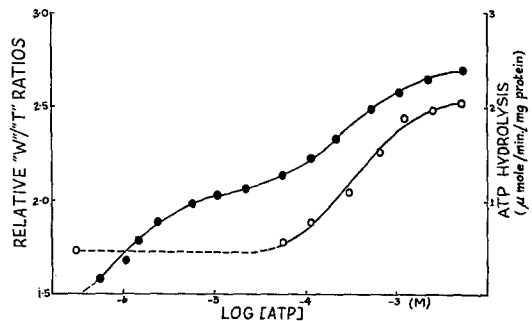

Figure 7. Relative ratios between "W" and "T" amplitudes in the EPR spectra of SR labelled with probe "B" (○), and rates of ATP hydrolysis (●), as functions of ATP concentrations. EPR measurements as described in Fig. 1, at 25° C. ATP hydrolysis measured in the presence of an ATP regenerating system, as described in the section on methods.

hydrophobic interactions in the interior regions of the protein, promoting an alteration of the native structure. Furthermore, deoxycholate effectively separates the lipid and protein components of SR; this separation itself may be sufficient to allow structural modifications in the protein phase, if lipoprotein association is an important structural determinant. Lastly, with the assumption that the membrane is constituted by repeating units,[1] it has been proposed that the conformational state of the units is conditioned by a cooperative effect of their reciprocal association, which would be expected to be seriously affected by the presence of deoxycholate.

The gross change induced on the spectra of spin labels "A" and "B" by denaturing agents such as urea and guanidine (Fig. 1) is expected, considering their interference with the protein intramolecular hydrogen bonds. It is important, however, to notice the lack of comparable effect of guanidine on label "C", as shown by the resolution of the two components of the high field nitrogen lines. This indicates that protein denaturation does not significantly modify the environment of label "C", and supports the idea that the label is in a region of low viscosity lipids.[8]

Binding of Nucleotides

ATP, ADP and ITP modify the EPR spectra of SR labelled with probe "B", conferring a greater mobility to the label. This indicates that the local environment surrounding a class of —SH groups undergoes a transformation related to the binding of these nucleotides. The transformation is localized, inasmuch as it does not involve spin label "A".

Both the adenosine (or inosine) and polyphosphate moiety are required for the effect of nucleotides, since PP has little effect, and AMP none. Apparently the effect is not due to the simple attribution of polyphosphate negative charges to the membrane, since 1 mM ATP is still effective in the presence of 5 mM $MgCl_2$. At these concentrations, Mg.ATP complex is the prevalent species and, based on an affinity constant of $10^{3.85}$ at neutral pH, it can be calculated that the free nucleotide is lower than 0·1 mM, which is its minimal effective concentration.

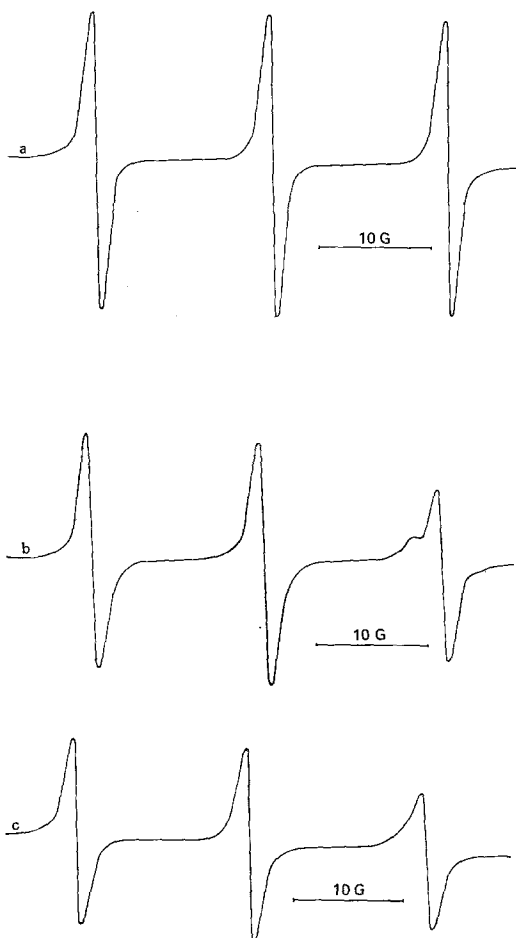The reversibility of the ATP effect is well demonstrated in Fig. 6, and proceeds at a

Figure 8. EPR spectra of probe "C" in aqueous phase (a), in the presence of SR (36 mg membrane protein/ml) (b), and in the presence of SR and 20 mM deoxycholate (c). The EPR spectra were obtained with 1·0 G Mod. Ampl., 70-mW power, 1-sec time constant, 50 G/8 min field scan rate.

faster rate in the presence of Ca^{2+}, which is a specific activator of SR ATPase. Since ADP is also effective, nucleotide dephosphorylation ultimately depends on myokinase, which is present in SR with approximately one-hundredth specific activity, as compared to ATPase.

Assuming that the mobilization of label "B" induced by ATP is due to a localized conformational change, it is important to consider if any relation exists between this effect of ATP and the regulation of ATPase activity. A comparison of the ATP concentrations required for ATPase activity and mobilization of label "B" (Fig. 7) may shed some light on this problem. A plot of ATPase activity as a function of substrate concentration shows a two-step sygmoid curve.[10,19] Since ATPase activity at low and high ATP concentrations obey otherwise identical kinetic parameters (Ca^{2+} activation, pH, temperature, —SH blocking) it has been concluded that Ca^{2+}-dependent ATP hydrolysis is catalyzed by only one enzyme of SR, which is further activated by high ATP concentrations.

The ATP concentrations required for mobilization of spin label "B" are in the same range and exhibit a behavior parallel to that of the second step in the plot of ATPase activity as a function of substrate concentration (Fig. 8). This suggests a relationship between ATPase activation by high (mM) ATP concentrations, and the ATP-induced mobilization of label "B" (i.e., conformational change).

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