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Conformational changes in the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum detected using phosphorescence polarization

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The technique of time-averaged phosphorescence has been used to study the interaction of calcium ions and ATP with the (Ca^{2+} + Mg^{2+})-ATPase in sarcoplasmic reticulum vesicles. The presence of excess calcium ions was found to cause a 20% decrease in the phosphorescence emission anisotropy. This is interpreted as being due to a conformational change in the protein and is supported by data from time-resolved phosphorescence measurements which also show a lowering of the anisotropy. This change in the decay of the emission anisotropy is associated with only minor changes in the rotational relaxation time of the protein and is again suggestive of a conformational change in the protein. In some cases ATP was also observed to lower the time-averaged phosphorescence anisotropy possibly via an interaction with the low-affinity regulatory site of the protein.

Introduction

The binding of calcium ions to the (Ca^{2+} + Mg^{2+})ATPase of sarcoplasmic reticulum has previously been shown to affect both the level of the intrinsic tryptophan fluorescence and the fluorescence yield of fluorescein isothiocyanate covalently linked to the protein [1–3]. These effects have been interpreted as arising from changes in the protein structure and this idea is supported by simultaneous changes in the availability of sulphydryl and carboxyl groups [4,5] and by increased stability against acid inactivation [6]. However, none of these studies have involved direct measurement of the enzyme's rotational mobility. In all cases a conformational change has been deduced based on the observation of a secondary effect.

We have recently described a novel technique, termed time-averaged phosphorescence polarization, for qualitatively measuring the rotational diffusion of intrinsic proteins in biological membranes [7]. This technique has the dual advantages of being both quick and easily performed on a commercially available luminescence spectrometer. In the present paper we report the application of this new technique to studies of the interaction of calcium ions and ATP with the (Ca^{2} + Mg^{2+})-ATPase of sarcoplasmic reticulum. Comparisons are also made between the time-averaged method and data obtained using time-resolved phosphorescence depolarization.

Abbreviations: (Ca^{2+} + Mg^{2+})-ATPase, calcium-dependent adenosine 5′-triphosphatase; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; ANS, 1-anilino-8-naphthalenesulfonate.

Materials and Methods

Sarcoplasmic reticulum was isolated from the white muscles of the back and hind legs of rabbits essentially as described by Nakamura et al. [8]. Labelling of the $(Ca^{2+} + Mg^{2+})$-ATPase with the triplet probe, erythrosin 5-isothiocyanate (Molecular Probes Inc. 24750 Lawrence Rd. Junction City, OR 97448) was performed by incubating the sarcoplasmic reticulum vesicles, suspended in 50 mM sodium phosphate buffer (pH 8.0), with an appropriate amount of probe to give a 1 : 1 stoichiometry of probe to protein. After incubation for 1 h at room temperature any unreacted probe was removed by centrifugation at $80\,000 \times g$ for 30 min and the pellet resuspended in sodium phosphate buffer (pH 8.0) containing 66% v/v glycerol and 0.5 mM EGTA. Other experiments have previously shown that, provided the probe solutions are freshly prepared, the proportion of probe non-covalently associated with the sarcoplasmic reticulum is negligible [11].

Typically 1 mg of protein was used for each time-averaged experiment and 0.3 mg for the time-resolved measurements. All the manipulations and incubations were performed under conditions of dim red light (Kodak Safelight Filter No. 1) or total darkness in order to prevent photo-oxidative damage to the protein [9,10]. Prior to performing the rotational diffusion measurements, the samples were thoroughly deoxygenated by passing nitrogen gas through them in order to prevent oxygen quenching of the triplet state.

Time-averaged phosphorescence polarization measurements were performed using a Perkin-Elmer LS-5 luminescence spectrometer as described elsewhere [7]. Two independent measurements of the emission anisotropy were taken for each sample. These values (appropriately corrected for errors introduced by non-ideality of the instrument optics) were then averaged to give the final result. The apparatus used for the time-resolved phosphorescence measurements was as described by Restall et al. [11] with the exception that the photomultipliers used were EMI type D554. The laser-induced phosphorescence was collected from both sides of the sample cuvette, perpendicular to the direction of the laser beam and focussed through polarizers aligned parallel on one side and perpendicular on the other to the electric vector of the laser excitation. This was oriented in the vertical direction normal to the plane of excitation and observation, yielding signals $I_{VV}(t)$ and $I_{VH}(t)$, respectively. Collection of these data sets was alternated with that of the same two emission components excited by horizontally polarized light. The collection of these two components ($I_{HV}(t)$ and $I_{HH}(t)$) was essential to allow correction for differences in the registration of the orthogonally polarized emission components.

Labelling of sarcoplasmic reticulum membranes with 1,6-diphenyl-1,3,5-hexatriene was carried out by incubating the vesicles at room temperature for 1 h with sufficient probe to give a probe-lipid ratio of 1 : 500. An Elscint model MV1a microviscosimeter was used to measure the fluorescence polarization.

Results

Excitation of erythrosin isothiocyanate with a brief pulse of light of suitable wavelength, results in transient emission of phosphorescence at wavelengths greater than 650 nm [7,12]. If the excitation beam is plane polarized the emission may also be polarized. The extent of this polarization can be measured by monitoring the level of light emission both parallel (I_{VV}) and perpendicular (I_{VH}) to the vertically polarized excitation beam. Calculation of the emission anisotropy $R = (I_{VV} - I_{VH})/(I_{VV} + 2I_{VH})$ allows the degree of polarization to be quantified.

In a situation where the probe is free to rotate isotropically, such as when it is in solution, the observed anisotropy will decay to zero within a few nanoseconds. However, if the probe is attached to a relatively slow moving substrate, such as an intrinsic protein, then the decay of anisotropy will be much slower. In this case the anisotropy will decay on a timescale of a few hundred microseconds and will also show more complex kinetics [13–15]. By studying the time-dependence of the emission anisotropy, information can be derived both about the dynamics and the degree of orientational constraint on the protein movement.

The technique of time-averaged phosphorescence polarization relies upon exciting the sample with a flash of polarized light and then

integrating the intensities of phosphorescent light emitted both parallel and perpendicular to the polarization plane of the exciting light. The integration is performed over a preset time interval corresponding to the time over which the anisotropy is expected to decay and from the integrated intensities the anisotropy is calculated as described above. Theoretically, if the sample is excited with horizontally polarized light, the emission signals observed (I_{HV} and I_{HH}) should be identical and their ratio $G = I_{HH}/I_{HV}$ independent of both the decay of the excited state population and its depolarization. Experimentally, the G-value is seldom equal to 1 and therefore the anisotropy has to be corrected for this instrumental factor, i.e. $R = (G \cdot I_{VV} - I_{VH})/(G \cdot I_{VV} + 2I_{VH})$.

In this study we have examined the effect of calcium ions on the phosphorescence emission anisotropy from erythrosin isothiocyanate-labelled ($Ca^{2+} + Mg^{2+}$)-ATPase in sarcoplasmic reticulum membranes using both time-averaged and time-resolved techniques. Fig. 1 shows the temperature dependence of the time-averaged phosphorescence anisotropy both in the presence and absence of calcium ions. The addition of excess calcium clearly results in a lowering of the anisotropy over the temperature range examined. The extent of the effect was found to vary from sample to sample but the addition of calcium typically resulted in the anisotropy being decreased by about 20% (S.E. = 2.39). The reversibility of the effect was demonstrated by adding excess EGTA to chelate the calcium present. Under these conditions the anisotropy returned to the value observed prior to the addition of calcium. The presence of magnesium ions was not observed to cause any detectable change in the phosphorescence anisotropy either by itself or in the presence of calcium.

The addition of 1 mM *N*-ethylmaleimide, the thiol blocking reagent, to the sarcoplasmic reticulum was sufficient to completely abolish the lowering of the anisotropy by calcium. This is in accord with previous studies [1,2], which have shown *N*-ethylmaleimide to be an effective inhibitor of any observed calcium effect on the ($Ca^{2+} + Mg^{2+}$)-ATPase.

In order to ensure that the observed change in the emission anisotropy was not due to calcium ions affecting the lipid fluidity and hence having only an indirect effect on the protein mobility, diphenylhexatriene fluorescence polarization measurements were carried out. The results of these measurements, both in the presence and absence of calcium ions, are presented in Fig. 2. Over the temperature range examined no significant effect of adding calcium ions on the lipid fluidity could be detected. 66% v/v glycerol in the buffer was not observed to cause any change in the diphenylhexatriene fluorescence polarization either in the presence or absence of free calcium ions.

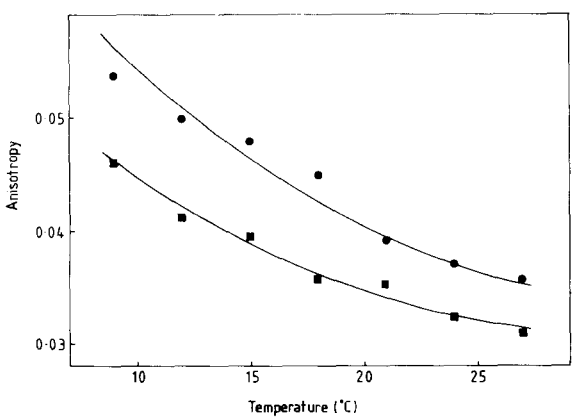

Fig. 1. The temperature dependence of the time-averaged phosphorescence anisotropy of the (Ca^{2+}-Mg^{2+})-ATPase protein, measured for sarcoplasmic reticulum vesicles in 50 mM sodium phosphate buffer (pH 8.0) containing 66% v/v glycerol and 0.5 mM EGTA, in the absence of exogenous Ca^{2+} (●) and in the presence of 0.55 mM Ca^{2+} (■).

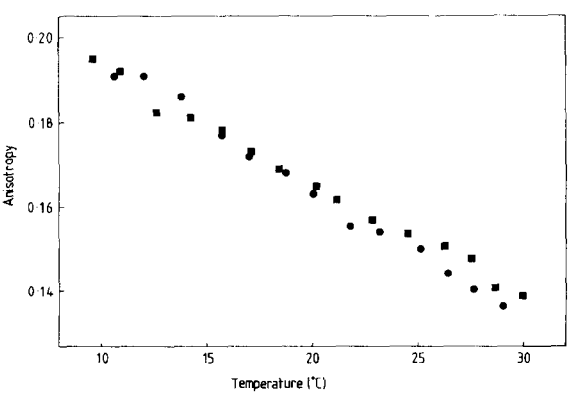

Fig. 2. The temperature dependence of the fluorescence anisotropy of diphenylhexatriene incorporated into sarcoplasmic reticulum vesicles in 50 mM sodium phosphate buffer (pH 8.0) containing 0.5 mM EGTA, in the absence of exogenous Ca^{2+} (●) and in the presence of 0.55 mM Ca^{2+} (■).

The effect of adding 5 mM ATP on the time-averaged phosphorescence anisotropy was also investigated. With some preparations of reticulum, a decrease in the anisotropy was also observed but the magnitude of the effect was typically lower (15%, S.E. = 2.26) than that observed with calcium. Other preparations of sarcoplasmic reticulum were found to give no change in emission anisotropy on addition of 5 mM ATP yet responded to calcium ions in the usual manner.

The $(Ca^{2+} + Mg^{2+})$-ATPase has two sites for the interaction of ATP, a high-affinity catalytic site (K_d about 10 μM) and a lower affinity regulatory site (K_d in the millimolar range) [16]. The addition of 5 mM ATP could, in princple, be affecting either or both sites. However, it is known that the binding of probes such as fluorescein isothiocyanate inhibits the interaction of ATP with the high-affinity catalytic site [17] by linking to a particular lysine residue [18]. In view of the close structural and chemical similarity between fluorescein isothiocyanate and the erythrosin isothiocyanate probe used in these studies, it seems likely they would bind to the same site. Consequently, the ATP may not be able to interact with the high-affinity site and the observed effect of 5 mM ATP on the phosphorescence polarization would result from its interaction with the regulatory site.

In order to test this hypothesis, the effect of ATP at micromolar concentrations on the phosphorescence polarization was investigated. No detectable change was observed at or below a concentration of 10 μM. Upon increasing the ATP levels to 1 mM, decreases in the phosphorescence polarization were again observed to occur. Arev and co-workers [19], in studies of the interactions of cations and nucleotides with the $(Ca^{2+} + Mg^{2+})$-ATPase using the fluorescence probe, 1-anilino-8-naphthalenesulfonate (ANS) have reported that the presence of 3 μM valinomycin inhibits the regulatory effect of millimolar levels of ATP but does not affect the high-affinity catalytic site. In our investigations we could not detect any effect of valinomycin on the interaction of millimolar levels of ATP with the $(Ca^{2+} + Mg^{2+})$-ATPase.

When ATP is added (giving a final concentration of 5 mM) to a system in which excess calcium ions are already present, a further decrease in the anisotropy is observed. This cumulative effect is also apparent if excess calcium ions are added to a system containing 5 mM ATP. The addition of further aliquots of calcium, raising the concentration of free calcium ions to millimolar levels, was found to have no further effect on the emission anisotropy.

The lowering of the phosphorescence anisotropy by calcium ions was studied in more detail by the technique of time-resolved phosphorescence polarization. This method differs from time-averaged phosphorescence in that the decay of emission anisotropy is followed with time by rapid sampling (every μs) over a discrete time interval. In contrast to the simpler time-averaged technique which provides an average anisotropy resulting from the integrated phosphorescence intensities observed over the measurement interval, this method allows a complete picture of the emission anisotropy to be obtained.

The detailed analysis of time-resolved phosphorescence polarization measurements is capable of yielding much information about the molecular dynamics [11]. In this particular study we have employed a simplified analysis whereby the time-dependent emission anisotropy due to the complex segmental movement of the protein has been analysed in terms of an equation comprising two exponentials plus a constant. The results of this

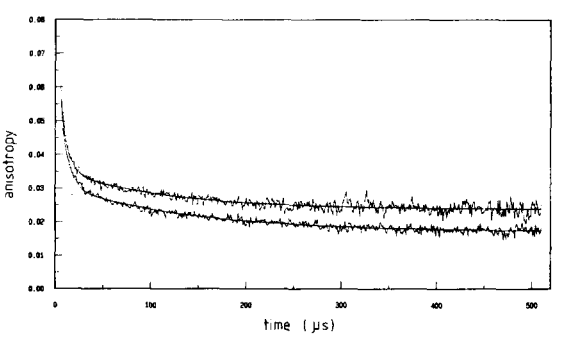

Fig. 3. The time-dependent phosphorescence anisotropy for erythrosin isothiocyanate-labelled $(Ca^{2+} + Mg^{2+})$-ATPase measured for sarcoplasmic reticulum vesicles in 50 mM sodium phosphate buffer (pH 8.0) containing 66% v/v glycerol and 0.5 mM EGTA in the absence of exogenous Ca^{2+} (upper trace) and in the presence of 0.55 mM Ca^{2+} (lower trace). The solid lines through the data represent the best fit to the equation: $R(t) = \beta_1 \exp[-t/\phi_1] + \beta_2 \exp[-t/\phi_2] + \beta_3$

TABLE I

Results of non-linear least-squares analysis of the time-resolved phosphorescence polarization data obtained for erythrosin isothiocyanate-labelled ($Ca^{2+} + Mg^{2+}$)-ATPase in the absence and presence of free calcium ions according to:

$$R(t) = \beta_1 \exp[-t/\phi_1] + \beta_2 \exp[-t/\phi_2] + \beta_3$$

	β_1	ϕ_1 (μS)	β_2	ϕ_2 (μS)	β_3	R_0
$-Ca^{2+}$	0.0608	6.4	0.0119	110	0.0238	0.0965
$+Ca^{2+}$	0.0520	7.5	0.0141	134	0.0171	0.0832

analysis, together with the experimental data are shown in Fig. 3 for both the sample in the presence of 0.5 mM EGTA (upper trace) and after addition of 0.55 mM Ca^{2+} (lower trace).

Once again a lowering of the anisotropy is clearly observed and this correlates well with the results obtained using the time-averaged methods. The parameters generated from the results of the best fit to the equation are summarized in Table I and show that the lowering of the anisotropy caused by the presence of calcium ions does not result from an increase in molecular mobility. This is apparent from the relatively small change in the ϕ terms which are actually observed to marginally increase. Instead, the change in the anisotropy decay curve is observed to arise from a change in the magnitude of the preexponential β terms. Since these parameters are determined by the degree of motional constraint imposed on the probe relative to the rotational diffusion axis, the results suggest a change has occurred in the conformation of the protein such that the portion of the protein on which the probe is located has now assumed a new orientation.

Discussion

Earlier studies of the interaction of calcium ions with the ($Ca^{2+} + Mg^{2+}$)-ATPase of sarcoplasmic reticulum have usually been interpreted in terms of a conformational change in the protein [1–3]. However, all of these methods rely on detecting some secondary effect which is believed to have resulted from the proposed conformational change. In this paper we have presented direct evidence of a calcium-induced conformational change in the ($Ca^{2+} + Mg^{2+}$)-ATPase using a technique which directly monitors the mobility and conformation of membrane proteins.

Changes in mobility cannot be distinguished from changes in conformation using time-averaged phosphorescence alone as averaged emission anisotropy is affected by both factors. Analysis of the data obtained from the time-resolved method, however, shows that this decrease in emission anisotropy is due to conformational changes, which affect the pre-exponential decay forms. Changes in the rotational mobility of the protein would change the kinetics of the anisotropy decay, that is the ϕ terms, whereas the initial (R_0) and final ($\beta_3 = R_\infty$) anisotropy values would be expected to be unaltered.

The data of Figs. 1 and 3 clearly show the lowering of the phosphorescence emission anisotropy which occurs when free calcium ions are present. In normal preparations of sarcoplasmic reticulum, the anisotropy values found in the absence of EGTA are typically observed to be closer to the values seen in the systems to which excess calcium ions have been added. This suggests that the small amounts of endogenous calcium ions present in the preparation are sufficient to induce the conformational change and therefore the affinity of the calcium binding site must be high.

With reference to the reaction scheme of De Meis and Vianna [20] shown in Fig. 4 for the

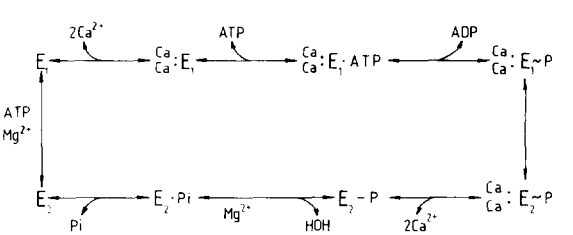

Fig. 4. Scheme demonstrating intermediates in the hydrolysis of ATP by the ($Ca^{2+} + Mg^{2+}$)-ATPase of sarcoplasmic reticulum according to De Meis and Vianna [20].

hydrolysis of ATP coupled to calcium translocation, the form of the enzyme responsible for the lower anisotropy terms must be the $2Ca \cdot E_1$ form. This is apparent since the lowering of the anisotropy occurs in the absence of ATP and furthermore, since the presence of the isothiocyanate probe blocks the binding of the ATP to the high affinity catalytic site, the enzyme is unlikely to be able to progress to the $2Ca \cdot E_1ATP$ form.

It has previously been reported [21] that as well as a regulatory site for ATP, the $(Ca^2 + Mg^{2+})$-ATPase also possesses a Ca^{2+}-binding site distinct from the high-affinity site. This site has been proposed to slightly accelerate phosphoenzyme hydrolysis. Higher concentrations of Ca^{2+} (> 2 mM) have been found to inhibit this reaction. Our results have indicated that raising the level of free calcium ions to millimolar concentrations has no additional effect on the emission anisotropy. It would appear therefore, that the anisotropy change observed is not dependent upon the hydrolysis of phosphoenzyme and would, instead, seem to reflect the E_2 to E_1 conformational change.

The form of the enzyme responsible for the high phosphorescence anisotropy is more difficult to predict since it could, in theory, be either the E_1, E_2, $E_2 \cdot P_i$ or the $E_2 \cdot P$ intermediate or indeed some weighted average of all these forms. The high levels of P_i present in our system resulting from the use of phosphate buffers might be expected to hold the enzyme in one of the E_2 forms. Under these circumstances the addition of calcium ions (or ATP) would favour the transition to the E_1 forms. This would be analogous to the change from the high (E_2) to low (E_1) fluorescence forms of ANS-labelled ATPase reported by Arav et al. [19].

The interaction of ATP with the $(Ca^{2+} + Mg^{2+})$-ATPase is not so clearly defined as it is with calcium. The presence of the erythrosin probe is expected to block the interaction of ATP with the high affinity catalytic site and consequently no effect should be observed. This is found to be the case with concentrations of ATP below 10 μM. At higher concentrations of ATP however, a lowering of the emission anisotropy is found. This is possibly due to interaction of the ATP with the lower-affinity regulatory site and may be associated with the E_2 to E_1 transition. However, it is clear that the E_2 to E_1 transition can occur in the absence of ATP since calcium would be unable to interact with the protein in the absence of ATP if this were not the case.

The absence of any inhibition of the interaction between millimolar ATP and the $(Ca^{2+} + Mg^{2+})$-ATPase by valinomycin should not be taken as evidence for the lack of interaction between ATP and the low-affinity binding site. In the studies reported by Arev et al. [19], valinomycin was shown to block the regulatory effect of millimolar ATP on the hydrolysis of ATP and calcium transport. However, the mechanism for this inhibition is not clear and may involve a more indirect effect on the protein rather than a simple blocking of the binding of ATP to the regulatory site.

When both ATP and calcium ions are added to the $(Ca^{2+} + Mg^{2+})$-ATPase, two distinct decreases in the phosphorescence emission anisotropy are seen, irrespective of the order in which the two ligands are added. If both ligands are causing the same conformational change (i.e., from the E_2 to E_1 form), then it is apparent that neither Ca^{2+} or ATP alone is capable of converting all of the protein to the E_1 form. Such a hypothesis is difficult to explain in terms of simple equilibria as depicted in Fig. 4 since it would be expected that a sufficiently high concentration of either calcium ions or ATP would be capable of producing the same effect. This appears not to be the case.

An alternative possibility may be that Ca^{2+} and ATP are affecting the protein in different ways. This may be the most plausible explanation of the observed results, particularly as in some preparations of sarcoplasmic reticulum no change in the emission anisotropy was observed following the addition of 5 mM ATP. If a portion of the $(Ca^{2+} + Mg^{2+})$-ATPase were present in an aggregated form which dissociated on addition of ATP then this could explain the lowering of the emission anisotropy. It would also explain the cumulative effect of ATP and calcium ions since the two ligands would be having separate, unrelated effects rather than affecting the same equilibrium process.

These studies have shown how the technique of time-averaged phosphorescence polarization can be used to study changes in the $(Ca^{2+} + Mg^{2+})$-ATPase induced by calcium ions and ATP. The calcium-induced change detected appears to be

associated with the E_2 to E_1 transition and as such does not represent the change in the enzyme responsible for calcium translocation directly. However, it may be related to the transition of the enzyme after calcium translocation has occurred. It is hoped that by studying such conformational changes in protein structure, clues may be obtained as to the mechanism of cellular ion transport.

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