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Ellipticity changes of the sarcoplasmic reticulum Ca^{2+}-ATPase induced by cation binding and phosphorylation

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The sarcoplasmic reticulum (SR) Ca^{2+}-ATPase is a member of the 'P-type' class of cation transport ATPases which form a covalent phosphorylated intermediate. It has been proposed that during ion transport, these proteins cyclically adopt two major enzymatic states E_1 and E_2, that are related to two essential conformations of the protein. By the use of especially sensitive circular dichroism (CD) instrumentation it is shown here that Ca^{2+} addition induces 5% or 2.5% increases in Ca^{2+}-ATPase ellipticity at 225 nm in the absence or in the presence of Mg^{2+}, respectively. Furthermore, a 2% change in the same direction was observed when the enzyme was phosphorylated with P_i in the absence of Ca^{2+}. These results suggest that the $E_1 \Longleftrightarrow E_2$ transition and the E_2-P formation are associated with structural changes of the polypeptide backbone structure of the calcium pump protein.

Sarcoplasmic reticulum; ATPase, (Ca^{2+}-Mg^{2+}); Circular dichroism

1. INTRODUCTION

The classical E_1–E_2 reaction cycle [1] describes a reaction scheme for 'P-type' transport ATPases. For the Ca^{2+}-ATPase, it is proposed that (i) in the E_1 form, the Ca^{2+}-ATPase has two Ca^{2+} high-affinity sites exposed to the cytoplasmic side of SR and can be phosphorylated by ATP to form the E_1-P intermediate, and (ii) in the E_2 form the Ca^{2+} binding sites are inaccessible to the external surface and the enzyme can react with inorganic phosphate P_i to form the E_2-P phosphorylated intermediate. It is under this last conformation that the transport sites become accessible from the lumen of the SR. One of the strongest supports for the $E_1 \Longleftrightarrow E_2$ scheme has been the detection of intrinsic fluorescence changes associated with transition between enzymatic states of the Ca^{2+}-ATPase [2]. From the analysis of the Ca^{2+}-ATPase sequence [3] and from the observation of tryptophanyl fluorescence quenching induced by membrane-soluble agents [4,5] it is very likely that most of the 13 tryptophan residues of the protein are located in transmembrane α-helical segments of the protein. Discrimination of individual tryptophan residues cannot be established and the changes observed may be due, either to modifications of the overall protein structure, or to small local re-adjustments induced by the proximity between the cation binding sites and the tryptophan residues. This latter hypothesis is supported by the fact that the calcium binding sites are proposed to be located within the transmembrane segments of the protein [6].

A few authors have tried to use other spectroscopic methods to detect Ca^{2+}-ATPase conformational changes. Arrondo et al. [7] used infrared spectroscopy and reported the detection of a difference between the E_1 and E_2 conformations, which they concluded from the appearance of a new α-helical substructure in the presence of EGTA + vanadate which stabilizes a conformation analogous to the E_2-P state. A few attempts have been made to use CD spectroscopy [8–10]. These authors conclude that no changes in CD spectra were detectable except irreversible effects observed for detergent-solubilized ATPase [8,10]. It is interesting to note that, on the contrary, significant differences in ellipticity were found between the E_1 and E_2 conformations of the Na/K-ATPase [11].

2. EXPERIMENTAL

We have investigated the CD signal of Ca^{2+}-ATPase at 225 nm using an optical system of higher sensitivity than conventional CD spectrophotometers. This system is associated either to a stirred cuvette holder permitting substrate injection during the recording (steady-state recordings) or to a stopped-flow system (transient state recordings). The light source consisted of a 150 W Xe(Hg) arc lamp and a single grating monochromator. It was connected to the cuvette assembly via a tapered optical fiber. Cuvette assembly was composed of a polarizer, a modulator, a stirred cuvette holder and a photomultiplier positioned against the cuvette. Small size of the beam passing through the cuvette gave an angle of acceptance of the output light of 80° thus minimizing light scattering effects [12]. Substrates were injected with Hamilton syringes in the cuvette under stirring without

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opening the cuvette holder. Mixing time for a 2 ml filling was around 1 s. The signal detected by the photomultiplier was processed with a lock-in amplifier, filtered at 3 s time constant, digitalized and analysed with a microcomputer software (Bio-Kine from Bio-Logic Co). The level of stray light was measured to be less than 1%. This system was calibrated by measuring the CD signal from a 0.1% solution of isoandrosterone in dioxane at 304 nm. Stopped-flow instrument was the Bio-Logic SFM-3 fitted with a 2 mm light path cuvette (FC-20). The integration time constant was 10 ms and data were analysed with the Bio-Kine software. Furthermore, the Ca^{2+}-ATPase CD spectrum was recorded stepwise from 200 to 250 nm, it was compared with that obtained by using a JY/DC-III spectropolarimeter. All results were found to be equivalent to published spectra of Ca^{2+}-ATPase [8,9].

3. RESULTS AND DISCUSSION

We first measured the steady-state changes induced by calcium binding and dissociation (Fig. 1). A calcium-dependent reversible change in ellipticity was observed which corresponds to an increase of the absolute value of the ellipticity of about 5% for the E_1 state (Ca^{2+} bound) as compared to the E_2 state (free enzyme). Sample absorbance was recorded at the same time as CD. No absorbance changes were observed for the $E_1 \rightarrow E_2$ transition and only small and slow change for the $E_2 \rightarrow E_1$ transition.

Ca^{2+}-ATPase represents about 75% of the SR proteins; the only other protein present in significant amount is calsequestrin which represents an average of 5% in weight. It is therefore certain that the entire CD change observed is due to the Ca^{2+}-ATPase. The rate constants of these transitions were obtained with the stopped-flow configuration. The values found (see Fig. 2) are in good agreement with those obtained from intrinsic fluorescence studies performed under the same experimental conditions [13,14].

The calcium concentration dependence of the ellipticity increase is shown in Fig. 3A. It is correctly fitted with a model assuming two cooperative binding sites with a 0.06 μM apparent dissociation constant. This is in good agreement with the variation of intrinsic fluorescence of Ca^{2+}-ATPase measured under similar conditions [2,15]. Therefore, it is very likely that the ellipticity increase observed upon the addition of calcium corresponds to the structural change associated with the opening of the two high-affinity binding sites.

It has been proposed [14,16] that Mg^{2+} may be able to bind to one of the high-affinity Ca^{2+} binding site. At variance with Ca^{2+}, binding of Mg^{2+} to this rapidly accessible cation binding site (termed 'f' site in [14]) does not induce the full $E_2 \rightarrow E_1$ transition, the second slow cation binding site ('s' site) remaining inaccessible. We show here that Mg^{2+} ions induce a partial increase of ellipticity of the Ca^{2+}-ATPase with an apparent dissociation constant of 0.6 mM (Fig. 3B). Addition of Ca^{2+} in the presence of saturating concentrations of Mg^{2+} induces a further increase of ellipticity which then becomes equal to that reached using Ca^{2+} only (Fig. 3C). Accordingly, analysis of the calcium concentration dependence of this further ellipticity change indicates an increase of the apparent dissociation constant for Ca^{2+} in agreement with a competitive inhibition by Mg^{2+} (K_i = 0.7 mM). The amplitude of the ellipticity change induced by Mg^{2+} equals one-half of that associated with

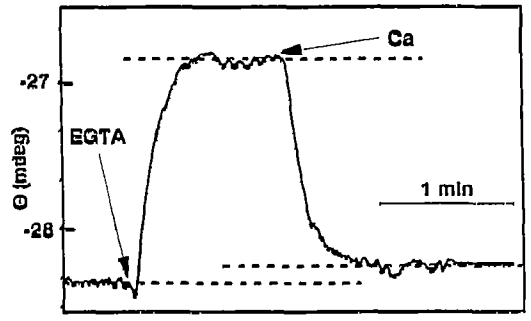

Fig. 1. Steady-state recordings of calcium-induced ellipticity change of Ca^{2+}-ATPase in SR vesicles at 225 nm. Ca^{2+}-ATPase concentration was 50 μg/ml. Vesicles were suspended in 2 ml of 20 mM MOPS-KOH buffer pH 7.0, 100 mM KCl, 10 μM Ca^{2+} at 25°C. At the beginning of the recordings, the Ca^{2+}-ATPase was in the E_1 state, transition to E_2 was induced by the injection of 5 μl of 100 mM EGTA (final concentration 250 μM). Reversal to E_1 was induced by the injection of 2 μl of 1 M $CaCl_2$ (final total $CaCl_2$ concentration 1 mM).

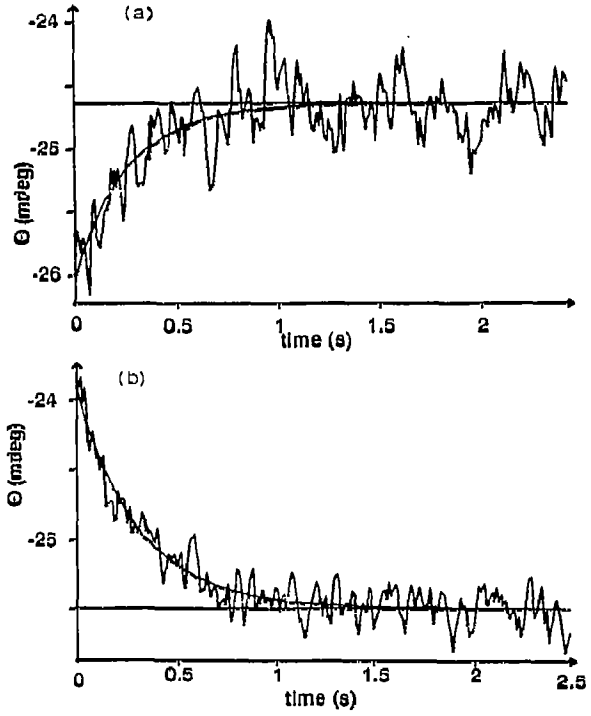

Fig. 2. Time-resolved measurements of ellipticity changes measured by stopped-flow. The traces shown are averages of 50 shots. Experiments were performed under conditions where the $E_1 \Leftrightarrow E_2$ transconformation was relatively slow: T_c = 10°C, 40 mM MES-Tris buffer, pH 6.2, no potassium and no magnesium present in the solution. (a) Dissociation of calcium. Initial conditions were: syringe 1, 1 mM EGTA; syringe 2, unused; syringe 3, 400 μg/ml proteins in 200 μM Ca^{2+}. Mixing was 1/1. The fit corresponds to a rate constant of 3 s^{-1}. (b) Binding of calcium. Initial conditions were: syringe 1, 500 μM Ca^{2+}; syringe 2, unused; syringe 3, 400 μg/ml proteins in 400 μM EGTA. Mixing was 1/1. The fit corresponds to a reaction rate of 3.3 s^{-1}.

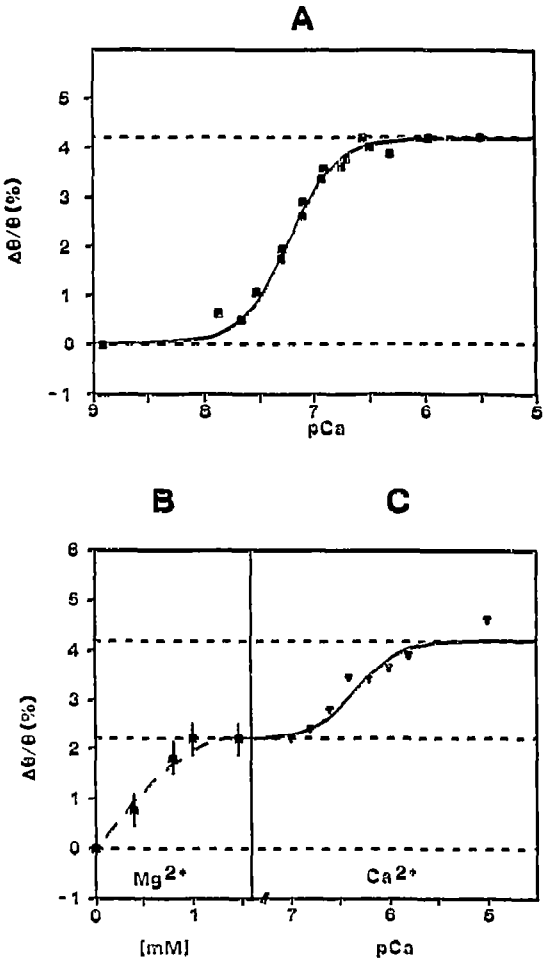

Fig. 3. (A) Calcium concentration dependence of the Ca^{2+}-ATPase CD signal at 225 nm. Conditions were as described in Fig. 1 except for the $CaCl_2$ and EGTA concentrations which were adjusted to give the indicated free calcium concentration (calculated as described in [21]). The experimental data were simulated using a simple calculation assuming the binding of calcium to two cooperative sites of maximum interaction: $\Delta CD = \Delta CD_0 \cdot [Ca]^2 / (K_d^2 + [Ca]^2) \cdot \Delta CD_0$ being the maximum amplitude of the CD change and K_d the apparent mean dissociation constant of the calcium binding sites. The best fit was obtained for $\Delta CD_0 = 4.2\%$ and $K_d = 0.06\,\mu M$, corresponding to the line drawn under the data points. (B) Magnesium concentration dependence of the Ca^{2+}-ATPase CD signal at 225 nm in the absence of calcium. Experimental conditions were as described in Fig. 1 except for the presence of 500 μM EGTA in the buffer. Apparent dissociation constant for magnesium was estimated at 0.6 mM. (C) Calcium concentration dependence of the Ca^{2+}-ATPase CD signal in the presence of 5 mM Mg^{2+}. The experimental data were simulated as described in (A) above, except that the apparent dissociation constant used was $K'_d = K_d \cdot (1 + [Mg]/K_{Mg})$. The line under the data points was obtained using $\Delta CD_0 = 2.1\%$, $K_d = 0.06\,\mu M$ and $K_{Mg} = 0.7$ mM.

the entire $E_2 \rightarrow E_1$ transition induced by calcium. Thus we propose that the ellipticity change induced by Mg^{2+} only, is the result of the structural modification induced by the opening of the 'f' site.

Lastly, an intermediate level corresponding to the phosphoenzyme E_2-P can be identified by CD. The existence of an E_2-P conformational state was already detected by intrinsic fluorescence measurements [17] and is shown here to represent a distinct structural state (Fig. 4).

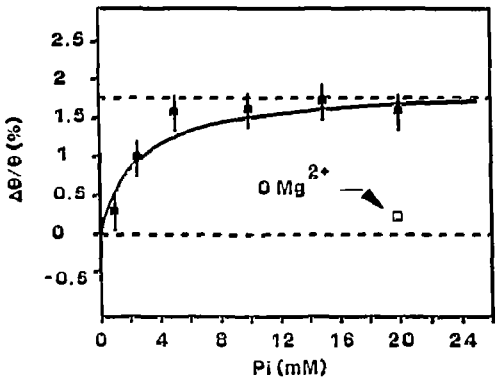

Fig. 4. Relative changes in the intensity of the Ca^{2+}-ATPase CD signal at 225 nm induced by inorganic phosphate in the absence of calcium (E_2-P state). Experimental set-up was as described under Fig. 1. The buffer was 40 mM MES-Tris, pH 6.2, 500 μM EGTA, 20 mM $MgCl_2$. As indicated a control experiment was performed with no added $MgCl_2$. The line under the experimental data corresponds to a fit assuming a single site for P_i with an apparent dissociation constant of 2.2 mM.

In their studies, Csermely et al. [9] reported a detection limit around 1 to 2% of ellipticity change mainly because of reproducibility between two samples. These authors, as well as Nakamoto and Inesi [8], made their measurements in the presence of millimolar concentrations of Mg^{2+}. As shown here, the maximum amplitude of the CD change induced by Ca^{2+} in this case is 2.5%; it is even lower for the phosphorylation-induced change. This has prevented these authors to detect the changes reported here. From the present CD study we can conclude that significant changes of secondary structure occur with specific steps of the reaction cycle of Ca^{2+}-ATPase; the calcium (or magnesium)-bound conformation and the phosphorylated form showing an increased ellipticity as compared to that of the free and unphosphorylated enzyme. This increase is very likely due to a larger extent in α-helices. This result is in agreement with what has been observed for other Ca^{2+}-binding proteins or peptides [18–20].

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