Retention of ellipticity between enzymatic states of the Ca^{2+}-ATPase of sarcoplasmic reticulum

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Circular dichroism spectra in the peptide region were obtained from the Ca^{2+}-ATPase of sarcoplasmic reticulum, to establish whether transitions of intermediate states of the enzyme cycle are accompanied by large changes of secondary structure. Since membrane-bound ATPase was used to avoid denaturation, absolute estimates of secondary structural content could not be obtained, due to light scattering interference. Nevertheless, it was possible to demonstrate unambiguously that nearly constant ellipticity is retained by the enzyme following enzyme transitions produced by calcium binding or phosphorylation, even though conformational changes are revealed by other structural probes under the same conditions. We conclude that the conformational changes involved in the long-range reciprocal influence of calcium and phosphorylation sites are related to ligand-induced displacements of amino acid residues which in turn produce reorientation of whole peptide segments of the ATPase protein. This is contrasted by the behavior of calmodulin which undergoes a definite change in ellipticity upon calcium binding.

Circular dichroism *Active transport* *Ca^{2+}-ATPase* *Sarcoplasmic reticulum*

1. INTRODUCTION

ATP-dependent calcium uptake SR vesicles was first described by Hasselbach and Makinose [1,2], and by Ebashi and Lipman [3], and attributed by the first 2 authors to active transport of Ca^{2+} across the SR membrane. Kinetic studies of this system have demonstrated that active transport is operated by a calcium-dependent ATPase through formation of a phosphorylated intermediate (reviews [4–10]). In fact, the basic mechanistic feature of the pump is a destabilization of the calcium-enzyme complex by enzyme phosphorylation at the catalytic site [6,7]. Since the calcium and catalytic sites do not overlap [7] and vectorial transport requires reorientation of the calcium bound, in addition to a reduction of the binding strength [11], we expect that coupling of catalysis and transport occurs through transitions of intermediate enzyme states accompanied by specific structural changes. In the simplest of cases we may consider the 3 enzyme states corresponding to (i) the free enzyme, (ii) the enzyme-calcium complex, and (iii) the phosphoenzyme intermediate. The transition associated with binding of calcium to the free enzyme manifests cooperativity and is required for ATP utilization by the enzyme. On the other hand, the phosphoenzyme intermediate (formed either by transfer of the ATP terminal phosphate, or directly through utilization of P_i in the reverse direction of the catalytic cycle) is the species exhibiting the transformation of the calcium sites which is required for active transport. Several studies (see table 1) suggest that significant structural changes are produced specifically by calcium binding and formation of the phosphoenzyme intermediate. An obstacle to a detailed elucidation of these putative changes is the lack of three-dimensional crystals of the SR ATPase which would be suited to high-resolution diffraction

Abbreviations: CD, circular dichroism; $C_{12}E_8$, dodecyl octaethylene glycol monoether; Mops, 4-morpholinepropanesulfonic acid; SR, sarcoplasmic reticulum

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analysis. However, other methods may be used to infer if transitions of enzyme states are accompanied by major reorganization of secondary structure, segmental protein reorientation or displacement of relatively few amino acid residues. A method assessing overall secondary structure is the measurement of ellipticity by CD. CD spectra of the SR ATPase have previously been reported [12–17] generally in the presence of detergents and sucrose. Andersen et al. [16] and Fronticelli et al. [17] reported decreases in total ellipticity and therefore shifts in secondary structure content upon removal of Ca^{2+} from the enzyme. Because these measurements were done with detergent-solubilized ATPase, it is not clear whether these changes are simply due to denaturation or correspond at least in part to specific enzyme transitions.

Here, we have utilized CD measurements to determine the extent of reorganization of secondary structure during the previously discussed enzyme transitions, and have considered these findings along with other types of structural information, with respect to the mechanism of active transport.

2. MATERIALS AND METHODS

SR vesicles were prepared as described [18] and the Ca^{2+}-ATPase purified by the method of Meissner et al. [19].

Purified calmodulin from bovine testis was kindly provided by Dr R.F. Steiner of the University of Maryland, Baltimore County.

CD spectra were taken on a Jasco J-40A spectropolarimeter utilizing a 1 cm square far-UV quartz cuvette. For shorter light path length, a quartz insert was utilized to decrease the effective path to 0.1 cm. The cuvette temperature was maintained at 25°C.

Experiments were carried out in the buffers described in the figure legends. Blank spectra were taken in the absence of protein for all conditions and subtracted from the protein spectra. Protein concentration was 0.1 mg/ml when a 1.0 cm light path was used, or 1 mg/ml with a 0.1 cm light path.

Ca^{2+}_{free} concentrations were determined by iterative calculations based on the algorithm of Fabiato and Fabiato [20].

Phosphoenzyme intermediate from $^{32}P_i$ in conditions identical to those of the CD measurements was determined as described [21].

SR phospholipids were extracted with chloroform-methanol as described by Bligh and Dyer [22] and then dried and resuspended by sonication in aqueous buffer under N_2 gas to prevent oxidation of the phospholipids.

3. RESULTS

3.1. *Circular dichroism of Ca^{2+}-ATPase*

CD spectra of the Ca^{2+}-ATPase from SR previously reported were generally done with buffers containing mild detergents such as deoxycholate or $C_{12}E_8$ and high sucrose concentrations of 0.2–0.6 M [12,14,15,17]. However, the enzyme tends to denature when solubilized even in mild non-ionic detergents such as $C_{12}E_8$ and especially in the absence of Ca^{2+} [23,24]. The detergent solubilization creates varying rates of inactivation depending on the incubation conditions which results in complex kinetics of ellipticity changes upon addition or removal of Ca^{2+} or substrates [17]. The extent of ellipticity change seen upon removal of Ca^{2+} from $C_{12}E_8$-solubilized enzyme is seen in fig.1 which is similar to that reported by LeMaire et al. [15] and Fronticelli et al. [17]. The purpose of this work was to determine if this change is due to a transition related to the catalytic and transport cycle, or is rather due to denaturation. For this reason, we also determined CD spectra of the Ca^{2+}-ATPase still associated with its native phospholipids [19]. A typical CD spectrum (fig.1) of the purified ATPase without solubilization is compared to spectra obtained with solubilized ATPase. The lower ellipticity and less distinct 210 nm minimum are a result of absorption flattening and dispersion distortions caused by light scattering by the vesicles in suspension [13]. Because of the scattering artifacts, reliable estimates of secondary structural content from the CD measurements are not possible.

Despite these limitations, total ellipticities of various enzymatic states can still be compared to evaluate qualitatively changes in structure. To demonstrate that changes in ellipticity can still be detected in these conditions, we have used calmodulin (which has a well documented change in total ellipticity upon binding of Ca^{2+} [25]) to test

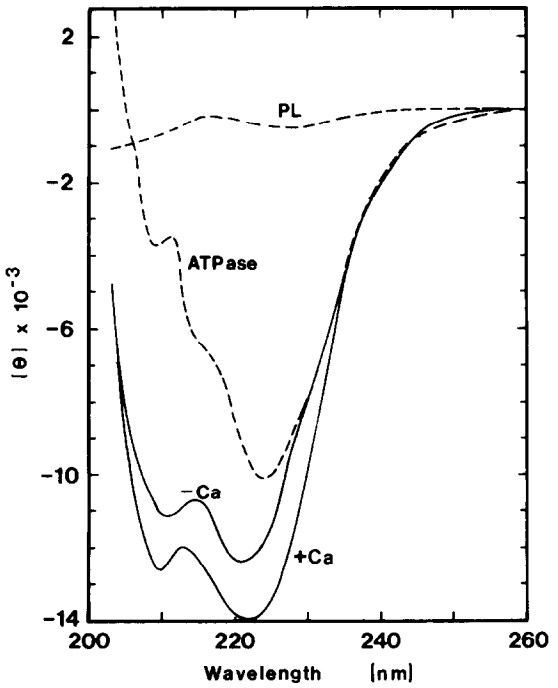

Fig.1. CD spectrum showing molar ellipticity of Ca^{2+}-ATPase in the absence or presence of $C_{12}E_8$ detergent. 0.1 mg/ml purified Ca^{2+}-ATPase [19] or 0.06 mg/ml SR phospholipids (PL) were suspended in 5 mM Mops, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 6.8, with (——) or without (– – –) 2 mM $C_{12}E_8$. 1.0 mM EGTA (0.01 μM Ca^{2+}_{free}) was added as noted. Optical path length was 0.10 cm. Temperature was 25°C.

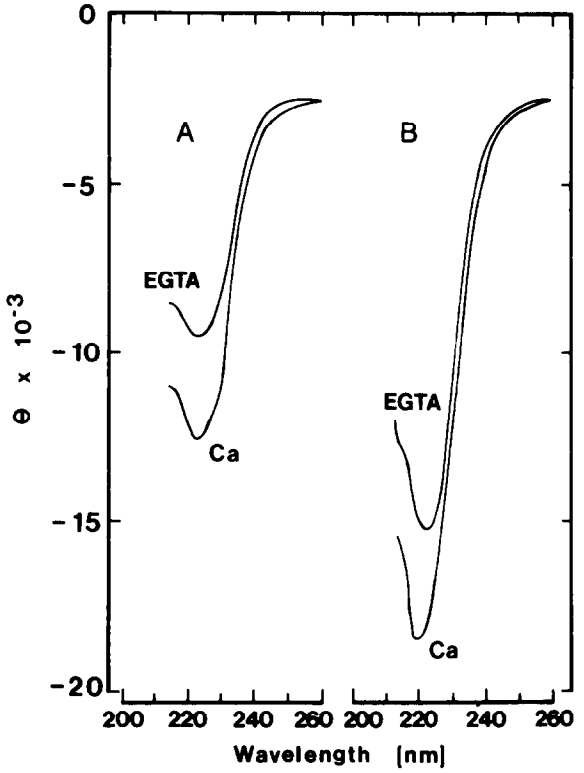

Fig.2. Measurement of ellipticity of calmodulin in the presence of vesicular Ca^{2+}-ATPase. The ordinate is not corrected for molar residue concentration. (A) 0.01 mg/ml calmodulin was suspended in 5 mM Mops, 0.5 mM EGTA, pH 6.8, in a 1.0 cm double cuvette at 25°C. 0.6 mM $CaCl_2$ was added to give the lower curve. (B) Identical to (A) except 0.01 mg/ml Ca^{2+}-ATPase vesicles in the same buffer was added to the second 1.0 cm chamber of the cuvette so that the light passed through the calmodulin sample first and then through the suspension of vesicles.

whether we could still measure the induced changes after passing the light through a suspension of ATPase vesicles. As seen in fig.2, the degree of ellipticity change measured at 222 nm upon addition of Ca^{2+} is still detected in the presence of vesicles even though the overall shape of the CD spectrum is quite different.

We also note that the circular dichroism seen in these spectra is due to the polypeptide and not to the phospholipids. In agreement with Lenard and Singer [26], CD spectra of extracted SR phospholipids. In agreement with Lenard and Singer [26], CD spectra of extracted SR phospholipids alone, at the equivalent concentration of phospholipids estimated for the ATPase vesicles (35% by dry wt, [27]), do not have a significant signal within the wavelength range of interest (fig.1).

3.2. *Effect of Ca^{2+} binding and phosphorylation on the ATPase CD spectrum*

We then measured the ellipticity of non-solubilized ATPase in the presence of either EGTA, or Ca^{2+}, or P_i, which stabilize the enzyme in 3 distinct states: free enzyme, enzyme-calcium complex and the phosphoenzyme intermediate complex. These 3 states represent the minimum number of expected enzyme conformations during catalysis and transport.

In the presence of enough Ca^{2+} (0.1 mM free) to saturate completely the high-affinity transport sites [28] the CD spectrum appears essentially identical to the enzyme incubated with EGTA (fig.3A

and B). This is in agreement with the observations of Mommaerts [12] on membrane-bound ATPase, but in contrast to the behavior of the enzyme solubilized with $C_{12}E_8$ [15,17]. In the case of the detergent-solubilized enzyme, the removal of Ca^{2+} results in a decrease of total ellipticity [16,17]. Fronticelli et al. [17] attributed such a decrease to denaturation occurring more quickly in Ca^{2+}-free, destabilized enzyme. We are in agreement with this interpretation since we found that the ellipticity decrease upon removal of Ca^{2+} from the solubilized enzyme is irreversible (not shown). In contrast, the membrane-bound ATPase is stable over an extended period of time with respect to both enzyme activity and total ellipticity, regardless of the presence of absence of Ca^{2+}.

The effect of formation of the covalent phosphoenzyme intermediate on the CD spectra was studied by incubating membrane-bound (i.e. non-solubilized) ATPase with P_i in the presence of EGTA ($[Ca^{2+}]_{free} = 1 \times 10^{-7}$ M) in the absence of KCl [29] and at pH 6.0. The conditions are known to maximize the phosphoenzyme level which was found to be 4.2 nmol/mg protein. (In separate experiments we found that the CD spectra of Ca^{2+}-incubated enzyme in either pH 7 plus KCl or pH 6 without KCl are identical.) It is shown in fig.3 that the CD spectrum of the phosphoenzyme is not significantly different from that of the free or Ca^{2+}-complexed enzyme.

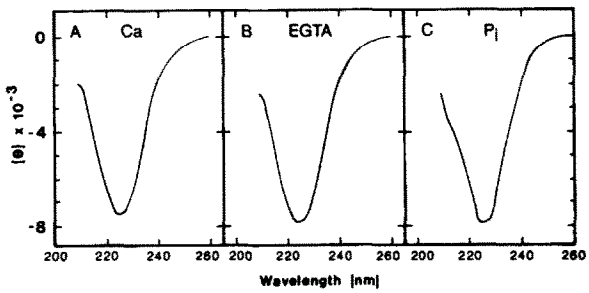

Fig.3. CD spectrum showing molar ellipticity of Ca^{2+}-ATPase incubated in EGTA, Ca^{2+} or P_i. 0.01 mg/ml purified Ca^{2+}-ATPase was suspended in either 5 mM Mops, 80 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, pH 6.8 (B), or 20 mM H_3PO_4, 20 mM $MgCl_2$, and 1 mM EGTA, with the pH adjusted to 6.0 with Tris (C). After obtaining CD spectra, 0.6 mM $CaCl_2$ was added to either medium; the result of spectrum (A) was identical in either condition. Phosphoenzyme level was 4.2 nmol/mg in the P_i-EGTA buffer. Optical path length was 1.0 cm. Temperature was 25°C.

4. DISCUSSION

From our results utilizing the membrane-bound SR ATPase, it is apparent that the transitions involved in the enzyme mechanism are not accompanied by major reorganizations of secondary structure. We have considered a minimum of 3 enzyme states in either of which the enzyme can be preferably placed by suitable manipulations of the reaction mixture. The ligand-free enzyme undergoes a transition which is produced by calcium binding to high-affinity transport sites and involves cooperative interactions [28]. Another transition accompanies formation of the phosphoenzyme intermediate. It is clear that these transitions (formations of enzyme-Ca complex and phosphoenzyme) are accompanied by distinct conformational changes which are integral parts of the enzyme mechanism as indicated by a variety of methods listed in table 1. Considering the lack of global changes in secondary structure, as indicated by the CD data presented here, it is apparent that the enzyme transitions involve displacement of amino acid residues which in turn produces segmental protein reorientation. In fact, a long-range conformational effect is required for the reciprocal influence of calcium and phosphorylation sites [6,7], since the calcium and phosphorylation sites do not overlap [7], and is likely to be operated by the peptide segment of residues 132–238 (from the structural model based on the complete primary structure of the ATPase [41]) including the second trypsin site (T_2). As discussed by Tanford [30], this

Table 1

Enzyme transition	Method	Reference
Enzyme to enzyme-Ca	tryptophan fluorescence	33
	ESR probe	34
	sulfhydryl reactivity	35,36
Enzyme to phosphoenzyme	TNP-ATP fluorescence	37
	low-angle X-ray diffraction	31
	formation of 2-dimensional crystals by incubation with vanadate (cf.[38])	32,39
	light scattering and fluorescence energy transfer during turnover	40

transition will probably affect the entire protein structure since protein structures, in general, are tightly coordinated. Thus, assuming no major perturbations of secondary structure, a series of parallel helices with the Ca^{2+} coordination groups devided amongst 2 or more of the helices (stalk region in the model of MacLennan et al. [41]) may undergo twisting and realignment of one of the helices [30] concomitant with utilization of ATP and formation of phosphoenzyme intermediate and resulting in alteration of the microenvironment of the bound calcium. This process does not require significant unfolding of helix or sheet structures.

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