Equilibrium between monomers and oligomers of soluble Ca^{2+}-ATPase during the functional cycle

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Received 1 July 1985

Molecular sieve HPLC shows that soluble sarcoplasmic reticulum Ca^{2+}-ATPase at low concentrations of the non-ionic detergent octaethylene glycol monododecyl ether exists as monomers in equilibrium with dimers and higher oligomers. Binding of vanadate or ATP as well as phosphoenzyme turnover shifts the equilibrium towards the monomer. This suggests that the Ca^{2+}-pump cycle can occur without transient self-association of Ca^{2+}-ATPase peptides.

High performance liquid chromatography *Detergent* *Ca^{2+} transport* *Vanadate* *Sarcoplasmic reticulum* *Protein-protein interaction*

1. INTRODUCTION

The Ca^{2+}-pump protein (Ca^{2+}-ATPase) of sarcoplasmic reticulum can be solubilized in monomeric form (M_r 115000) with retention of full enzymatic activity and free energy coupling [1–3]. It is not known whether oligomerization of peptide chains is required to provide the full structural basis for ion translocation through the membrane [4–6]. A conformation-dependent equilibrium between monomers and dimers of membrane-bound Ca^{2+}-ATPase is suggested by structure analysis of bidimensional crystals revealing monomeric or dimeric unit cells, dependent on whether lanthanide ions or vanadate are used to promote crystallization [7,8]. However, the functional significance of the 2 different crystal forms is difficult to evaluate due to the tight packing of proteins in the membrane.

Abbreviations: $C_{12}E_8$, octaethylene glycol monododecyl ether; Tes, *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; AMPPCP, adenosine 5′-[β,γ-methylene]triphosphate; E_1 and E_2, major conformational states of Ca^{2+}-ATPase

In preparations of Ca^{2+}-ATPase solubilized by non-ionic detergent, monomers, dimers, and higher oligomers of active protein exist in equilibrium at relatively low detergent to protein concentration ratios [3,9]. In this study we have examined the effect of ligand binding and protein conformational state on these equilibria by molecular sieve HPLC and analytical ultracentrifugation. HPLC provides a convenient tool for this purpose due to high separation efficiency and short elution time. We demonstrate a substrate-induced dissociation of Ca^{2+}-ATPase dimers and higher oligomers which is incompatible with induction of interpeptide associations during the turnover cycle.

2. MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle and purified as in [3]. For HPLC the membranes (4 mg protein/ml) were solubilized by addition of 10 mg $C_{12}E_8$ (Nikko Chemicals, Tokyo) per ml in either 20 mM Tes (pH 7.0), 0.1 M KCl, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$ or 20 mM Tes (pH 7.0), 0.1 M KCl, 10 mM $MgCl_2$, 1.0 mM EGTA, 100 μM Na_3VO_4 (in the

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00145793/85/$3.30

latter case the vesicles had been preincubated with vanadate for 1 h at 20°C before solubilization). For sedimentation velocity studies in the analytical ultracentrifuge 1 mg Ca^{2+}-ATPase/ml was solubilized by 1.5 mg $C_{12}E_8$/ml in th same buffers as for HPLC. The samples were centrifuged for 30 min in a Beckman airfuge at 130000 × *g* to remove unsolubilized material (constituting 20–30% of total protein irrespective of the presence or absence of vanadate).

Molecular sieve HPLC was performed at 20°C in a TSK G 3000 SW (7.5 mm × 60 cm) column (Toyo Soda) operated at a flow rate of 0.8 ml/min. Absorbance in eluted fractions was read automatically at 226 nm. The eluants contained either 20 mM Tes (pH 7.0), 0.1 M KCl, 10 mM $MgCl_2$, 1.0 mM EGTA and the $C_{12}E_8$ concentration indicated, or the same concentrations with further addition of 1.5 mM $CaCl_2$. In some experiments 1 mM ATP or the non-phosphorylating analogue AMPPCP was also present.

Ca^{2+}-ATPase activity was measured at 20°C on eluted fractions after dilution with buffer to obtain final concentrations of 0.1 M KCl, 20 mM Tes (pH 7.0), 0.1 mM free Ca^{2+}, 5 mM Mg^{2+}, 0.5 mg $C_{12}E_8$/ml, 1 mM phospho*enol*pyruvate and 0.2 mg pyruvate kinase (Sigma) per ml in a total volume of 500 μl. A procedure similar to that of Brotherus et al. [10] was followed. 100 μl of 30 mM MgATP was added to initiate the reaction, which was stopped 20 or 40 s later with 1 ml ice-cold 0.5 N HCl containing 30 mg ascorbic acid and 5 mg ammonium heptamolybdate. For colour development 1.5 ml of 20 mg sodium metaarsenite/ml, 20 mg sodium citrate/ml, 2% (v/v) acetic acid and 2 mg SDS/ml were added. The tubes were heated for 10 min at 37°C, and absorbance was read at 850 nm.

Phosphorylation assay, determination of protein concentration, and sedimentation velocity studies were performed as in [3].

3. RESULTS

Our previous analytical ultracentrifugation data have shown that a high [i.e. ≥5:1 (w/w)] detergent to protein concentration ratio is required to obtain a predominantly monomeric state of Ca^{2+}-ATPase solubilized by $C_{12}E_8$ [3,9].

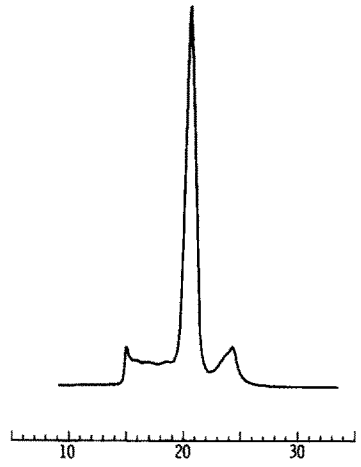


Fig.1. Molecular sieve HPLC of soluble Ca^{2+}-ATPase at 2 mg $C_{12}E_8$/ml. Ca^{2+}-ATPase was solubilized in the presence of 0.5 mM free Ca^{2+} and 500 μg was chromatographed as described in section 2 (eluant containing 0.5 mM free Ca^{2+}).

Fig.1 shows molecular sieve HPLC of soluble Ca^{2+}-ATPase at a $C_{12}E_8$ concentration of 2 mg/ml. The height of the major peak corresponds to a protein concentration of approx. 0.2 mg/ml. In the analytical ultracentrifuge this peak sediments as a single boundary with a sedimentation coefficient of 5.0 S, consistent with a Ca^{2+}-ATPase monomer [9]. The monomeric peak which possesses full Ca^{2+}-ATPase activity is preceded by a minor fraction of inactive aggregated material. Mixed micelles of phospholipid and detergent elute after the monomer.

At lower detergent to protein concentration ratios HPLC suggests the presence of self-associated Ca^{2+}-ATPase in equilibrium with monomer (fig.2). The figure shows chromatograms corresponding to detergent concentrations in the eluant ranging from below the CMC (0.05 mg $C_{12}E_8$/ml) up to 0.10 mg $C_{12}E_8$/ml. Elution positions of monomer and stable dimer are indicated. The latter assignment is based on sedimentation studies and gel electrophoresis of S-S cross-linked Ca^{2+}-ATPase (Andersen et al., in preparation). The oligomeric Ca^{2+}-ATPase in fig.2 was fully active (V_{max} 3.5–4.0 μmol/min per mg and phosphorylation level 4.5–5.3 nmol/mg). It was converted into the monomer when rechromatographed at a lower protein concentration (not

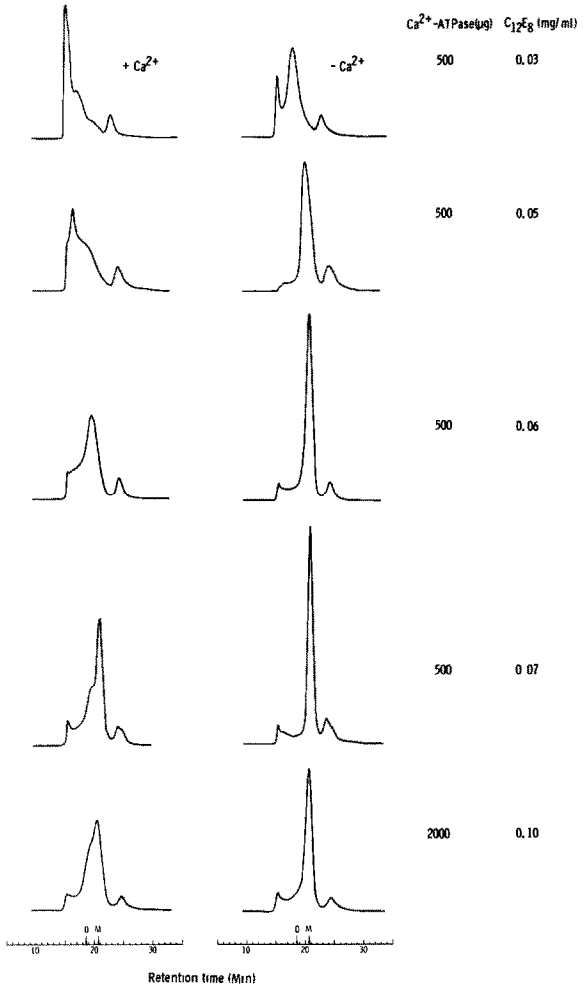


Fig.2. Effect of vanadate binding on self-association equilibria in soluble Ca^{2+}-ATPase at various detergent to protein concentration ratios. Vanadate-reacted Ca^{2+}-ATPase was solubilized in the absence of free Ca^{2+} and 500 μg or 2000 μg Ca^{2+}-ATPase (as indicated) was chromatographed in the presence (left) or absence (right) of Ca^{2+} as described in section 2. The detergent concentration in the eluant is indicated. Arrows show the elution positions of stable monomers (M) and dimers (D) (see text).

shown). Therefore the conditions in fig.2 are optimal for studying the effect of the functional state of Ca^{2+}-ATPase on reversible interpeptide associations.

On the left of fig.2 free Ca^{2+} was present in the eluant to promote dissociation of bound vanadate [$E_1(Ca^{2+})_2$ state], whereas Ca^{2+} was absent on the right of the figure. In the latter condition vanadate remained bound during HPLC as indicated by reversible inhibition of Ca^{2+}-ATPase activity and phosphorylation in the eluted fractions (table 1). The binding of vanadate is required to stabilize the E_2 conformation of soluble Ca^{2+}-ATPase.

It is apparent from fig.2 (cf. right and left) that transition from $E_1(Ca^{2+})_2$ to $E_2(V)$ shifts the self-association equilibria towards lower molecular mass complexes. At 0.03–0.05 mg $C_{12}E_8$/ml large oligomers of Ca^{2+}-ATPase emerging close to the void volume are dissociated into components eluting corresponding to trimer and dimer in equilibrium with some monomer. At 0.07–0.10 mg $C_{12}E_8$/ml Ca^{2+}-ATPase eluting corresponding to dimer is seen to be dissociated into monomer by vanadate binding.

When preincubation with vanadate in the absence of Ca^{2+} was omitted (i.e. 0.5 mM Ca^{2+} present during solubilization) the chromatograms obtained with excess Ca^{2+} in the elution buffer were almost identical to those of fig.2, left (not shown).

Fig.3 shows the influence of vanadate binding on sedimentation of soluble Ca^{2+}-ATPase in the analytical ultracentrifuge. The detergent to protein concentration ratio is only 1.5:1 (w/w). In the $E_1(Ca^{2+})_2$ state the boundary is drawn out corresponding to an equilibrium between monomers and oligomers of Ca^{2+}-ATPase. After binding of vanadate [$E_2(V)$] the enzyme sediments with a sharper boundary (average sedimentation coefficient 6.3 S). Thus the ultracentrifugation studies confirm the vanadate-induced dissociation of peptide-peptide interactions observed by HPLC.

It is seen from fig.4 that binding of ATP or the non-phosphorylating ATP analogue AMPPCP also promotes dissociation of self-associated Ca^{2+}-ATPase into monomers. The most homogeneous monomeric peak is seen with ATP in the presence of free Ca^{2+}. Phosphorylation and turnover take place during chromatography under this condition. A small fraction of the ATP concentration present in the column is hydrolyzed resulting in the appearance of a trough between the emerging Ca^{2+}-ATPase protein and total volume. ADP formed by hydrolysis of ATP gives rise to an absorbance peak at V_{tot}. ADP alone had a similar influence on the elution pattern to AMPPCP, but AMP and adenosine, which bind only weakly to Ca^{2+}-ATPase, had no discernible effects (not shown).

Table 1

Reactivation of vanadate-bound Ca^{2+}-ATPase after HPLC

	Control solubilized and chromatographed in the presence of 0.5 mM Ca^{2+}	Vanadate-reacted Ca^{2+}-ATPase chromatographed in the absence of Ca^{2+}	
		Assay initiated by addition of Ca^{2+} after ATP	Assay initiated by addition of ATP after 15 min preincubation with 0.5 mM free Ca^{2+}
Ca^{2+}-ATPase (μmol/min per mg)	3.8	0.25	3.15
Phosphorylation (nmol/mg)	4.6	0.05	4.1

HPLC was performed with 0.10 mg $C_{12}E_8$/ml in the eluant. Ca^{2+}-ATPase and phosphorylation were measured as described in section 2. When ATP is added before Ca^{2+}, bound vanadate does not dissociate during the assay [13]

4. DISCUSSION AND CONCLUSIONS

The Ca^{2+}-pump cycle in sarcoplasmic reticulum has been shown to involve at least 2 major conformational states of the Ca^{2+}-ATPase protein (E_1 and E_2) as indicated in scheme 1 [11,12]. E_1 has high-affinity binding sites for Ca^{2+} and is phosphorylated from ATP. E_2 has low-affinity Ca^{2+}-binding sites and reacts with inorganic phosphate. Both forms bind ATP although their affinity may differ [12,13]. Vanadate as a phosphate analogue binds exclusively to E_2. Struc-

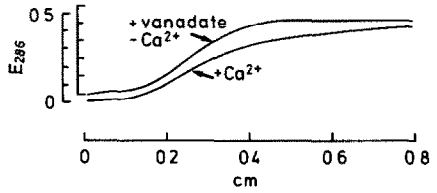


Fig.3. Effect of vanadate binding on sedimentation of soluble Ca^{2+}-ATPase at a low detergent to protein concentration ratio. Ca^{2+}-ATPase (1 mg/ml) was solubilized by 1.5 mg $C_{12}E_8$/ml in the presence of either Ca^{2+} or EGTA and vanadate as described in section 2. Sedimentation velocity was studied at 44000 rpm, 20°C, in a Beckman model E analytical ultracentrifuge. The scans shown were taken after 50 min.

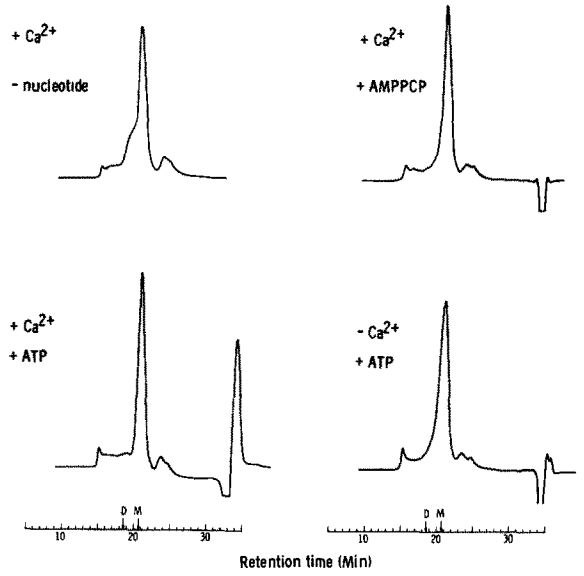
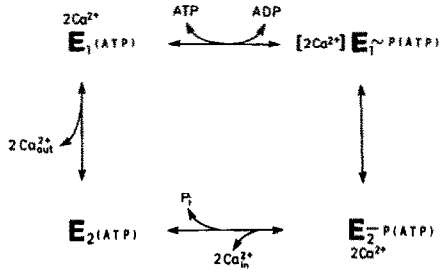


Fig.4. Effect of nucleotides and phosphorylation on self-association equilibria in soluble Ca^{2+}-ATPase at 0.10 mg $C_{12}E_8$/ml. Ca^{2+}-ATPase was solubilized in the presence of free Ca^{2+} and 500 μg was chromatographed as described in section 2. The eluant contained either 0.5 mM free Ca^{2+} or 1.0 mM EGTA alone, with or without further addition of 1 mM nucleotide as indicated.



Scheme 1

ture analysis has indicated that the E_1 conformation gives rise to a monomeric unit cell in 2-dimensional membrane crystals, while the vanadate-stabilized E_2 form crystallizes as a dimeric unit cell [7,8]. The present results show that for Ca^{2+}-ATPase solubilized in the active form at sufficiently low $C_{12}E_8$ to protein concentration ratios, monomers, dimers and higher oligomers are in equilibrium. We have demonstrated that the transition from $E_1(Ca^{2+})_2$ to $E_2(V)$ displaces this equilibrium towards the monomer. Therefore the possibility of a transient self-association occurring in relation to $E_1 \longrightarrow E_2$ conformational changes in the pump cycle [8,14] appears unlikely.

Binding of nucleotide in phosphorylating as well as in non-phosphorylating conditions was also found to promote monomerization (fig.4). With the medium composition studied here (pH 7.0, 0.1 M KCl, 0.5 mM Ca^{2+}) the predominant phosphoenzyme species present in the steady state is the E_1P form, which is supposed to occlude Ca^{2+} [15]. This intermediate gives rise to a homogeneous monomeric peak by HPLC at a detergent concentration which, in the absence of nucleotide, allows the formation of a considerable fraction of dimer (fig.4).

Our results lead to the conclusion that in all the major reaction intermediates of the Ca^{2+}-pump cycle peptide-peptide interactions are destabilized relative to the $E_1(Ca^{2+})_2$ state. This in conjunction with the report that the minimal asymmetric unit in Ca^{2+}-ATPase membrane crystals consists of a single polypeptide chain [8] provides strong support for the monomer as the functional unit in Ca^{2+} transport.

The mechanism by which binding of vanadate and nucleotides shifts the equilibrium between the various forms of soluble Ca^{2+}-ATPase remains to be clarified. Local charge effects as well as structure changes in the vicinity of the catalytic site may contribute. The same ligands which cause monomerization have been shown to protect the soluble protein against irreversible structure changes in the absence of Ca^{2+} [9]. Therefore a decreased exposure of hydrophobic residues to solvent, induced by stabilization of the functionally important protein conformations, may also be involved.

ACKNOWLEDGEMENTS

This research was supported by The Danish Medical Research Council, The P.C. Petersen Foundation and The Foundation for The Advancement of Medical Science. Liselotte Madsen is thanked for skilful technical assistance.

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