Solubilized monomeric sarcoplasmic reticulum Ca pump protein

Phosphorylation by inorganic phosphate

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Phosphorylation (by inorganic phosphate) of sarcoplasmic reticulum Ca pump protein has been studied in a detergent solution in which the protein has been previously shown to exist as a monomer. The course of the reaction is qualitatively similar to that observed for membrane-bound (possibly oligomeric) protein. In particular, the results indicate that alternation between the two principal conformational states of the Ca pump protein persists in the monomeric state, which suggests that the machinery for coupling of ATP hydrolysis to Ca^{2+} transport is intact. There are quantitative differences between monomeric and membrane-bound protein with respect to phosphorylation, but they are not necessarily related to the state of association.

Active transport *Free energy coupling* *Calcium ATPase*

1. INTRODUCTION

Definition of the minimal functional unit of the sarcoplasmic reticulum Ca pump protein is a major issue in relation to the mechanism whereby this protein couples ATP hydrolysis to active transport of Ca^{2+}. Mandatory requirement for an oligomeric structure has been suggested [1–4], but evidence to the contrary exists as well [5,6]. It is known that the Ca pump protein can be solubilized as a monomer by the nonionic detergent $C_{12}E_8$ without loss of ATPase activity, and with retention of the cooperative activation of this activity by Ca^{2+} [7–9]. Moreover, the method of active enzyme centrifugation has demonstrated that the monomer is the actual active species under these conditions [11]. These results suggest that one of the two principal conformation states of the protein (the E state in fig.1) is unaffected by solubilization as a monomer. On the other hand, the protein in $C_{12}E_8$ is structurally unstable when EGTA is added to reduce the concentration of free Ca^{2+} [8]. The native membrane-bound protein is converted to its alternate conformational state, the normal Ca^{2+} discharge state E′, when this is done [13,14], and structural instability upon EGTA addition thus raises the possibility that the normal E′ state may not be capable of existing when the protein is monomeric. Since alternation between the E and E′ states is a critical element of the coupling mechanism [15], this implies that the unaltered ATPase activity observed in $C_{12}E_8$ could represent uncoupled hydrolysis of the substrate.

Phosphorylation by inorganic phosphate, first reported in [15], provides a tool for investigating this question, because it represents a reversal of that part of the reaction cycle that normally occurs in the E′ state. Mg^{2+} is an obligatory constituent

Abbreviations: $C_{12}E_8$, dodecyl octaethyleneglycol monoether; SR, sarcoplasmic reticulum

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of the product (MgE'-P), and the equilibria involved in MgE'-P formation (fig.1) have been quantitatively evaluated for membrane-bound Ca pump protein in SR vesicles [17,18].

2. MATERIALS AND METHODS

Leaky SR vesicles and monomeric protein in $C_{12}E_8$ were prepared and characterized as in [11]. Solubilized preparations generally contain 4–9 mol residual phospholipid per mol protein, in addition to bound detergent. All soluble and vesicular preparations had ATPase activities of at least 16 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ (37°C) immediately before use and the functional unit of the soluble preparations remained monomeric in the presence of substrates (MG^+, Ca^{2+}, K^+, ATP) during ATP hydrolysis [11].

An early attempt to phosphorylate the solubilized protein with P_i proved unsuccessful [19], but authors in [20] were able to form monomeric MgE'-P in $C_{12}E_8$ in the presence of high concentrations of dimethyl sulfoxide. In our hands, dimethyl sulfoxide was not required. Phosphorylation was carried out essentially as in [18], at pH 6.2, 25°C, 0.2–0.4 mg/ml Ca pump protein, in the absence of KCl. Excess EGTA was present, except in the experiment describing Ca^{2+} inhibition. The above conditions (pH 6.2, excess EGTA) destabilize the solubilized monomer and ultimately lead to inactivation followed by aggregation. To minimize the effects of destabilization, phosphorylation conditions were rapidly induced by a step addition of reagents to the temperature equilibrated monomer and the reaction quenched after 10 s incubation, therby permitting $<5\%$ inactivation of the ATPase. Phosphorylation levels remained constant for 10–$\geq$60 s. Measurements for solubilized protein were carried out in 0.9 mM $C_{12}E_8$, and in the presence of 5–9% glycerol, derived from the solutions in which the protein was stored. (A similar level of glycerol was present in the experiments in which the monomeric state of the protein is $C_{12}E_8$ was established.) Glycerol was added to the same level in experiments with vesicular preparations. Authors in [21] have shown that 5–10% glycerol has a marginal promoting effect on MgE'-P formation in vesicles, far less than dimethyl sulfoxide, a result which we have confirmed.

3. RESULTS

Fig.1 shows the reaction scheme previously used for analysis of phosphorylation in SR vesicles [13,14]. E' is the thermodynamically favored state of the protein in the absence of ligands ($K_0 \gg 1$), i.e., state E is not involved when MgE'-P is formed from unliganded protein. Inhibition of MgE'-P formation by added Ca^{2+} or at high $[Mg^{2+}]$, however, involves cation binding sites in state E [13,14], so that studies of these inhibitions provide information about the E⇌E' transition for the monomeric protein.

Preliminary experiments showed that lower yields of phosphoenzyme were obtained in $C_{12}E_8$ than for vesicular protein at the same Mg^{2+} and P_i concentration. However, the pH dependence of MgE'-P formation for the monomeric protein is similar to what has been observed for the membrane-bound state [16], with an optimal yield near pH 6.2. All subsequent measurements were carried out at this pH.

Fig.2 shows that inhibition of MgE'-P formation by Ca^{2+} is qualitatively similar to inhibition observed for the vesicular protein [16], and in particular that it is a cooperative function of $[Ca^{2+}]$. The measured Hill coefficient (inset) is about 1.4, similar to that reported for equilibrium Ca^{2+} binding studies at pH 6 [22]. In terms of the scheme of fig.1, this implies that the equilibrium constant K_0 remains $\gg 1$ after solubilization. Fig.3 shows that the dual effect of $[Mg^{2+}]$, activation of MgE'-P

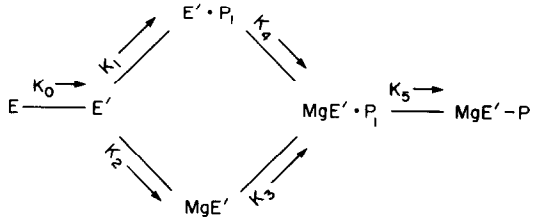

Fig.1. Reaction scheme for phosphorylation. E and E' refer to the two principal conformational states of the Ca pump protein. E is the state that binds ATP and cytoplasmic Ca^{2+} under physiological conditions, and E' is the state from which Ca^{2+} is normally discharged into the SR lumen (see [13] for full discussion). K_5 has a value near unity, which is why the maximal yield of MgE'-P is limited to a value near 0.5 even at saturating concentrations of P_i [13,18].

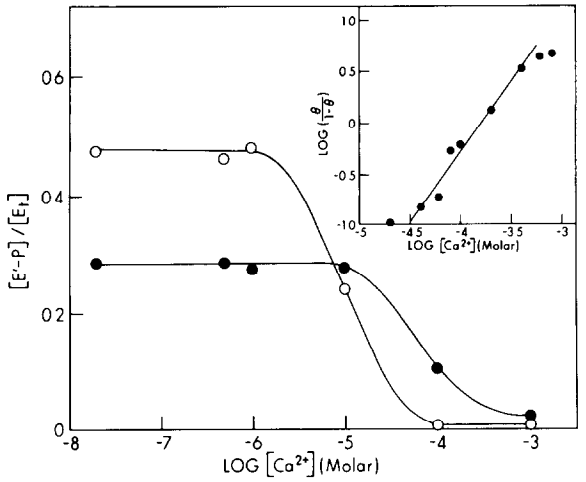

Fig.2. Ca^{2+} inhibition of MgE'-P formation at constant [P_i]. Open symbols represent leaky SR vesicles, filled symbols represent solubilized protein. The inset is a Hill plot of additional data obtained with the solubilized system over a narrower concentration range. The slope of the plot is 1.4.

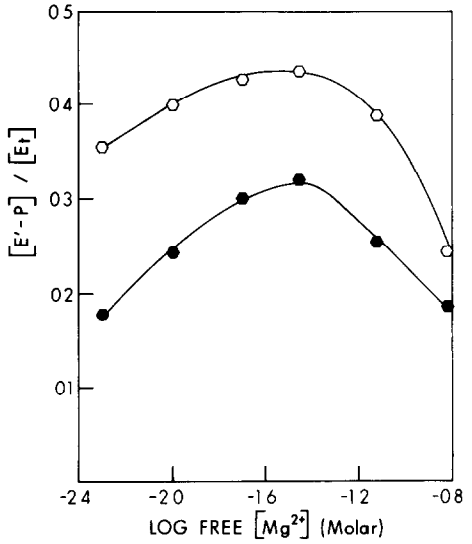

Fig.3. Mg^{2+} inhibition of MgE'-P formation at constant [P_i]. Open symbols represent leaky SR vesicles, filled symbols represent solubilized protein. All solutions contained 5 mM P_i. SD for data from 4 experiments was $<4\%$.

formation at low concentrations and inhibition at high concentrations [13], is also preserved. The inhibitory effect is again highly cooperative with respect to [Mg^{2+}], consistent with $K_0 \gg 1$.

To investigate the lower yields obtained with the monomeric protein, we studied the formation of MgE'-P as a function of [P_i] at a constant [Mg^{2+}] of 20 mM (fig.4A). It is evident that the difference between the two preparations is greatest at low [P_i] and that the yields approach equality at high [P_i]. Fig.4B shows a Scatchard plot of the same data. The intercepts *(I)* on the *x*-axis represent the maximal yields of MgE'-P at infinite [P_i], and are seen to have the same value within experimental error for both preparations. If each data set is separately analyzed by linear regression analysis, we obtain $I = 0.55 \pm 0.02$ and 0.51 ± 0.03, respectively. Another set of experiments (not shown) yielded $I = 0.49$ and 0.50, respectively. Our previous detailed study with leaky SR vesicles [18], in which both

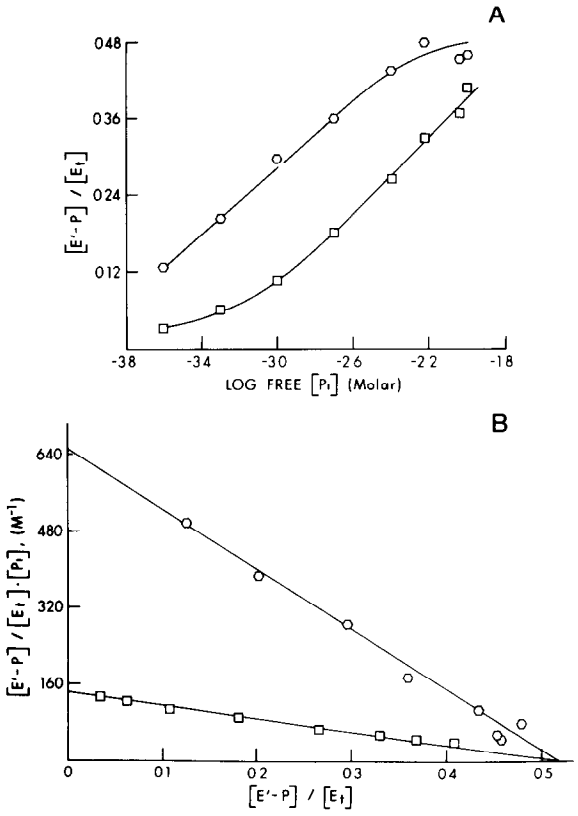

Fig.4. (A) MgE'-P formation at pH 6.2 at a constant Mg^{2+} concentration of 2 mM and variable [P_i]. Squares represent leaky vesicles, hexagons represent solubilized protein. SD for data from 4 experiments of this kind was $<4\%$. (B) Scatchard plot of the same data. The intercept on the *x*-axis represents the maximal amount of MgE'-P that can be formed at 20 mM Mg^{2+}.

$[Mg^{2+}]$ and $[P_i]$ were varied (but $[Mg^{2+}]$ was kept below the level at which inhibition sets in) gave $I = 0.48$. This result, with the aid of equations given previously ([18], see also [22]) demonstrates that the number of phosphorylation sites per protein molecule is unaltered by solubilization, but that the binding affinity for P_i is reduced. A similar result (same number of sites, but lower binding affinity) is obtained from a Scatchard plot based on the Mg^{2+}-dependence of phosphorylation, using the data of fig.3 below 20 mM Mg^{2+}, i.e., at Mg^{2+} concentrations too small for manifestation of the cooperative inhibitory effect.

4. DISCUSSION

The most important conclusion of this paper is that the E′ state unquestionably exists in the monomeric protein, and that it retains the ability to carry out the normal reactions of the E′ state. Phosphorylation by P_i proceeds qualitatively in the same way as for membrane-bound protein. Inhibition of phosphorylation by Ca^{2+} or by high concentrations of Mg^{2+} shows that the reversible interconversion of the E and E′ states is unimpaired. Since this interconversion is at the heart of the free energy coupling process [15,23], our results suggest that the molecular machinery for coupling is intact in the monomeric state. ATP synthesis from MgE′-P can also be carried out in the monomeric state [10,12,20]. The result is important for mechanistic considerations, because it shows that free energy coupling does not require protein-protein interaction, but it does not necessarily imply that the actual functional unit in the native SR membrane is monomeric.

We have also confirmed and amplified earlier demonstrations of differences between $C_{12}E_8$ solubilized monomeric Ca pump and membrane-bound (possibly oligomeric) protein in SR vesicles. Our results indicate that the monomeric protein contains the same binding sites for substrates and metal ions as the membrane-bound protein, but that ligand binding affinities in the E′ state are reduced. A similar result was previously reported for ATP binding in $C_{12}E_8$ solution. Ca^{2+}-induced ATP hydrolysis in the solubilized state resembles the same process in SR vesicles (even the maximal rates are the same), but a biphasic dependence on ATP concentrations for the membrane-bound protein, believed to reflect binding of ATP to the E′ state, is not seen in the same concentration range for the monomer [7,8]. These altered binding affinities, and the previously reported structural lability in the E′ state [6,8], need not be related to the state of association of the protein. They could reflect a specific lipid requirement for maintenance of the true native structure, or interference of the long polyoxyethylene polar groups of the detergent with the exposed polar portions of the protein, or inadequacy of the relatively short dodecyl chain of $C_{12}E_8$ for shielding of the normally membrane-associated part of the protein from the aqueous medium. The results obtained do not necessarily apply to detergents other than $C_{12}E_8$ that can solubilize the Ca pump protein without loss of ATPase activity.

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