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pH and ligand binding modulate the strength of protein–protein interactions in the Ca^{2+}-ATPase from sarcoplasmic reticulum membranes

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Abstract

The Ca^{2+}-ATPase from sarcoplasmic reticulum (SR) membranes couples the Ca^{2+} transport to ATP hydrolysis through phosphorylation in its cytoplasmic catalytic domain. Interactions between protein domains and the role of monomer–monomer interactions remain unclear. Here, we report a differential scanning calorimetric study of the thermal unfolding of this protein. In the pH range 6–8, thermal unfolding of the Ca^{2+}-ATPase in glycogen phosphorylase-free SR membranes shows a major endothermic peak with a critical temperature midpoint ranging between 51 and 55°C, depending on pH, Ca^{2+}, Mg^{2+}-ADP and KCl concentrations. The enthalpy change of the overall unfolding process ranged between 250 and 300 kcal/mol of Ca^{2+}-ATPase monomer. Thermal denaturation of the Ca^{2+}-ATPase in SR membranes is well fitted to an irreversible process that can be rationalized in terms of a non-two state process, N (native) $\rightleftharpoons$ I (intermediate) $\rightarrow$ D (denatured). Thermodynamic analysis show that this protein has a compact structure, implying a tight structural interconnection between catalytic and Ca^{2+} transport domains. The apparent cooperative unit, defined by the van 't Hoff enthalpy to the overall unfolding enthalpy ratio, increased from 1.1 at pH 6 to 1.8 at pH 8, showing that monomer–monomer interactions are stronger at weakly basic pH than at weakly acidic pH. While micromolar Ca^{2+} concentrations had only a weak effect on the cooperativity of the unfolding process, this is clearly increased by millimolar Mg^{2+}-ADP. In addition, high ionic strength lowered the apparent cooperative unit to approximately 1.0 in the pH range 6–8. Taken together, these results suggest that protein–protein interactions are altered by variables that modulate the catalytic activity of this enzyme.

Keywords: Ca^{2+}-ATPase; Differential scanning calorimetry; Ca^{2+}; Nucleotide; Thermal denaturation

1. Introduction

The Ca^{2+}-ATPase (EC 3.6.1.38) from sarcoplasmic reticulum (SR) couples hydrolysis of ATP to Ca^{2+} transport to the lumen of SR vesicles [1]. This protein belongs to E1/E2 ATPases [2] and Ca^{2+} binding shifts the E1/E2 equilibrium [3]. Ca^{2+} binding to the high affinity Ca^{2+} sites of the Ca^{2+}-ATPase has been shown to be strongly dependent on pH [3] and to increase the stability of this enzyme against thermal

Abbreviations: Ca^{2+}-ATPase, Ca^{2+}-dependent adenosine triphosphatase (EC 3.6.1.38); SR, sarcoplasmic reticulum; DSC, differential scanning calorimetry; ΔH, overall unfolding enthalpy; ΔH_v, van 't Hoff enthalpy; T_m, critical temperature midpoint; $C_{12}E_8$, octaethylene glycol dodecyl ether; NCD-4, *N*-cyclohexyl-*N'*-(4-dimethylamino-α-naphthyl) carbodiimide; TES, *N*-Tris[hydroxymethyl] methyl-2-aminoethanesulfonic; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; I.U., amount of enzyme that releases 1 μmol product per min

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denaturation, both in SR membranes and in purified Ca^{2+}-ATPase [4–7]. In addition, binding of Mg^{2+}-ADP and Mg^{2+}-ATP to the catalytic site also produces a large stabilization of purified Ca^{2+}-ATPase against thermal unfolding [6,7]. These results suggest a tight structural relationship between catalytic and transport domain in the Ca^{2+}-ATPase. Kinetic studies also support this conclusion [8,9]. In addition, X-ray diffraction analysis of oriented SR membranes revealed a compact three-dimensional structure of this protein in the SR membrane [10,11]. The high density of the protein in the membrane, 31 000–34 000 ATPases/μm^2, suggests that the protein is forming oligomers in the native membrane, and this is showed by freeze-fracture studies [12]. The role of these oligomers in the ATPase cycle and the possible modulation by ligand binding of subunit–subunit interactions that take place in these oligomers have been studied using electron paramagnetic resonance [13,14].

Differential scanning calorimetry (DSC) is a useful technique to study the structural organization of proteins [15]. Previous DSC studies of the Ca^{2+}-ATPase have shown that the thermal unfolding of this protein takes place between 47 and 55°C [7,16–20]. On the basis of the complex pattern of DSC thermograms of SR membranes, the existence of two different functional domains that unfold separately was suggested, being the one with the highest T_m a Ca^{2+} domain, which presumably contained the Ca^{2+} transport sites in the transmembrane domain of the protein [18]. In a later study, it was reported a $K_{0.5}$ value of 0.25 mM for the effect of Ca^{2+} at pH 7 on the denaturation process of this domain [20], far from the $K_{0.5}$ of Ca^{2+} binding to the high affinity sites (which is 0.1–0.2 μM at pH 7 [21]). It was suggested that Ca^{2+} binding to low affinity Ca^{2+} sites located in the transmembrane or stalk subdomains of the Ca^{2+}-ATPase, stabilized a putative transmembrane domain [20]. However, DSC studies with purified Ca^{2+}-ATPase revealed a simple endothermic peak for the thermal denaturation of the enzyme [7,16].

Glycogen phosphorylase is a major contaminant protein in SR membranes prepared following current protocols [22]. The presence of this enzyme largely alters the pattern of the denaturation process of SR membranes, because glycogen phosphorylase shows an exothermic peak that overlaps with the endothermic transition of the Ca^{2+}-ATPase [23]. When glycogen phosphorylase is removed from SR membranes, using the procedure described in [22], the calorimetric experiments carried out with phosphorylase-free SR membranes revealed a DSC pattern of denaturation simpler than previously observed [16,18] and closer to that reported for purified Ca^{2+}-ATPase [7,16].

We have previously demonstrated that pH modulates the conformation of the nucleotide binding domain of this protein, and that an increase of pH from 6 to 8 cannot be solely rationalized in terms of a E2–E1 conformational equilibrium [24]. In this paper we have assessed the thermal unfolding of the Ca^{2+}-ATPase in phosphorylase-free SR membranes, and its modulation by pH, Ca^{2+} and Mg^{2+}-ADP binding. We conclude that the protein unfolds as a compact structure following a kinetic process that can be explained with an algorithm of a non-two state process, modulated by pH, in which the intermediate state is likely related with oligomers dissociation.

2. Materials and methods

2.1. Preparation of sarcoplasmic reticulum membranes, purified Ca^{2+}-ATPase, and purified calsequestrin

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle (New Zealand white) as previously described [25]. Glycogen phosphorylase in the SR vesicles was removed using the procedure described in [22]. Ca^{2+}-ATPase was purified by the method of MacLennan [26]. Using SDS/gel electrophoresis (7–10% acrylamide) plus Coomassie blue staining [27], we calculated that the Ca^{2+}-ATPase is the 75–80% of the total protein in SR membranes and that the purity of the protein in the purified preparation was higher than 98%. Calsequestrin was purified by the method described in [28]. Protein concentration was measured following the method of Lowry et al. [29], using bovine serum albumin as a standard.

2.2. Ca^{2+}-ATPase activity measurements

Ca^{2+}-ATPase activity was measured spectrophotometrically using the coupled enzyme pyruvate kinase/lactate dehydrogenase assay [30], with the following reaction mixture at 25°C: 0.1 M TES/KOH (pH 7.45), 0.1 M KCl, 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 2.5 mM ATP, 0.42 mM phosphoenolpyruvate, 0.25 mM NADH, 7.5 I.U. pyruvate kinase and 18 I.U. lactate dehydrogenase. SR preparations showed less than 5% of Ca^{2+}-independent activity, measured in presence of 3 mM EGTA. Purified Ca^{2+}-ATPase showed negligible Ca^{2+}-independent ATPase activity. The ATPase activity of SR showed 4–5-fold stimulation upon addition of 4% (μg/μg of SR protein) of the Ca^{2+} ionophore calcimycin. The specific Ca^{2+}-ATPase activity of SR in presence of calcimycin and of Ca^{2+}-ATPase preparations ranged between 3–5 and 7–9 μmol ATP hydrolyzed per min per mg of protein at 25°C, respectively.

2.3. Differential scanning calorimetry measurements

Scanning calorimetry measurements were carried out using a differential scanning calorimeter MicroCal MC-2, operated at a scanning rate ranging from 10 to 90°C/h and under a nitrogen pressure of 1.5–2 kg/cm^2 during the scan. All the scans reported in this paper are representative of, at least, triplicate experiments carried out with triplicate preparations of SR membranes or purified Ca^{2+}-ATPase. The concentration of protein in the DSC experiments was 2 mg/ml. The buffer used in DSC experiments was 20% glycerol, 0.1 M KCl and 0.02 M TES/KOH (pH 7 or 8) and 20% glycerol, 0.1 M KCl and 0.02 M MES/KOH (pH 6). The amount of K^+ from the KOH used to adjust the pH was approximately 5 mM. The pH change of the solutions during the scans was always lower than 0.20 pH units, e.g. from 7.00 at 25°C to 6.85 at 60°C. Ca^{2+} and EGTA were added as indicated in the text. In presence of EGTA the free Ca^{2+} concentration was calculated using dissociation constants for the Ca^{2+}–EGTA complex of 3.85 10^{-5} M (pH 6), 3.94 10^{-7} M (pH 7) and 4.46 10^{-9} M (pH 8) [31]. The samples were degassed before loading the calorimeter. The analysis of the calorimetric data was carried out with the Origin software developed by MicroCal (Northampton, MA, USA) for the MC-2 differential scanning calorimeter. The thermal unfolding of a protein is a kinetic process [15] and the unfolding of the Ca^{2+}-ATPase in the SR membrane is appropriately described with the algorithm for a non-two state transition. Sánchez-Ruiz et al. [32] and Lepock et al. [33] developed the theoretical background for a non-two state unfolding process that conforms to the simple kinetic scheme $N \rightleftharpoons I \rightarrow D$, being N, I and D the native, intermediate and denatured protein states, and its scan rate dependence.

The curve fitting process gives the parameters: T_m, ΔH and ΔH_v. The fitting process used Marquadt methods based on non-linear least-squares, being improved the guesses for each parameter, and using an iterative process until there is no further improvement in the fit of the calculated to the experimental parameter as indicated by a minimum value of χ^2.

Baselines were taken as shown in Fig. 1 for typical raw data of SR membranes (trace a) and purified ATPase (trace b) at pH 7, in presence of 0.5 mM Ca^{2+}, and at a scanning rate of 30°C/h.

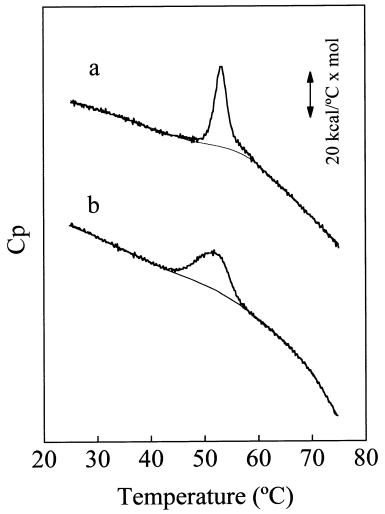

Fig. 1. Excess heat capacity (raw data) of SR membranes (a) and purified ATPase (b) at pH 7 versus temperature. Typical DSC record before baseline subtraction of SR membranes or purified ATPase (2 mg of protein/ml) in presence of 0.5 mM Ca^{2+}. Scanning rate, 30°C/h; buffer, 20% glycerol, 0.1 M KCl and 0.02 M TES/KOH (pH 7). Baselines calculated for both traces were drawn by the Origin software developed by MicroCal, (Northampton, MA, USA).

3. Results

3.1. Differential scanning calorimetry study of SR membranes

DSC thermograms of glycogen phosphorylase-free SR membranes at pH 7 in the temperature range 25–75°C and at a scanning rate of 30°C/h, show the presence of one major endothermic peak, both in presence of 0.5 mM (Fig. 2, trace a) and 2 nM (Fig. 2, trace b) free Ca^{2+} concentrations. Thermal unfolding of the SR membranes corresponds to the denaturation of the Ca^{2+}-ATPase, because the endothermic peak seen in DSC scans overlaps with the temperature range at which SR samples irreversibly lost the ATPase activity under the same conditions (buffer and heating rate) used in the calorimeter ([7] and data not shown). Additionally, thermal unfolding of the Ca^{2+}-ATPase is an irreversible process (Fig. 2, trace c), as previously shown [7,19].

Analysis of the DSC thermograms of SR membranes revealed that the algorithm for a two-state transition yielded a very poor fit of the data (dotted line over trace a, Fig. 2). The best fit of the data is obtained with an algorithm for a non-two-state transition (which completely overlaps the DSC trace). Table 1 lists the midpoint temperature of the endothermic peak (T_m) and the enthalpy of denaturation (ΔH) of Ca^{2+}-ATPase unfolding obtained from the analysis of DSC thermograms, of SR membranes at pH 6, 7 and 8, in presence of 0.5 mM Ca^{2+} or 2 mM EGTA, and with a scan rate of 30°C/h. In this pH range, only one major endothermic peak is observed in the DSC scans. These results show that the ATPase is thermally less stable at pH 6 than at pH 7–8

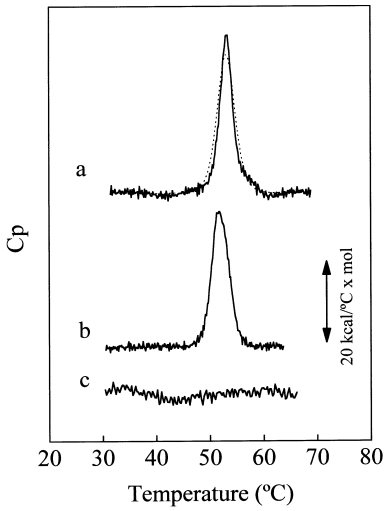

Fig. 2. Excess heat capacity of SR membranes at pH 7 versus temperature. Typical DSC recording of SR membranes (2 mg/ml) in presence of 0.5 mM Ca^{2+} (trace a) and in presence of 2 mM EGTA (2 nM free Ca^{2+} concentration) (trace b). Scanning rate, 30°C/h; buffer, 20% glycerol, 0.1 M KCl and 0.02 M TES/KOH (pH 7). Trace c is a re-scan of a sample heated up to 75°C. The results obtained by fitting the trace a to the algorithm for a two-state transition (dotted line) are also shown. The result obtained by fitting the trace a to the algorithm for a non two-state transition completely overlapped the DSC trace.

Table 1
Thermodynamic parameters of the thermal unfolding of Ca^{2+}-ATPase in SR membranes at different pH values

	SR membranes	T_m (°C)	ΔH (cal/g SR)	ΔH (kcal/mol)
pH 6	+0.5 mM Ca^{2+}	51.5 ± 0.2	2.40 ± 0.27	264 ± 39
	+2 mM EGTA	50.6 ± 0.3	2.50 ± 0.25	275 ± 31
	+2 mM EGTA/3 mM Mg^{2+}-ADP	53.5 ± 0.2	2.35 ± 0.26	259 ± 31
pH 7	+0.5 mM Ca^{2+}	53.5 ± 0.4	2.43 ± 0.30	267 ± 48
	+2 mM EGTA	52.6 ± 0.2	2.64 ± 0.23	291 ± 33
	+2 mM EGTA/3 mM Mg^{2+}-ADP	54.5 ± 0.6	2.65 ± 0.27	292 ± 39
pH 8	+0.5 mM Ca^{2+}	54.0 ± 0.1	2.54 ± 0.31	280 ± 50
	+2 mM EGTA	53.8 ± 0.1	2.22 ± 0.26	244 ± 47
	+2 mM EGTA/3 mM Mg^{2+}-ADP	54.7 ± 0.4	2.43 ± 0.19	267 ± 26

Scanning rate, 30°C/h; buffers, 20% glycerol, 0.1 M KCl and 0.02 M TES/KOH (pH 7 or 8); 20% glycerol, 0.1 M KCl and 0.02 M MES/KOH (pH 6). The conversion of ΔH values from cal/g of SR protein to kcal/mol of Ca^{2+}-ATPase monomer was made taking into account that 80% of the total SR protein is Ca^{2+}-ATPase (see Section 2), with a molecular weight of 110 kDa. Values are given as mean ± S.E.M., with $n \geq 6$.

(T_m values at pH 6 are 2.5–3°C lower than at pH 7–8). The thermal instability of the Ca^{2+}-ATPase induced by weakly acidic pH (6.0) cannot be attributed to a shift of the E1/E2 conformational towards the E2 conformation, because it is observed in presence of a Ca^{2+} concentration saturating the high affinity Ca^{2+} sites on the Ca^{2+}-ATPase (E1 state). In addition, the protein is less stable against thermal unfolding in presence of EGTA (free Ca^{2+} concentrations of 190, 1.98 and 0.02 nM at pH 6, 7 and 8, respectively) than in presence of Ca^{2+} concentrations saturating the high affinity Ca^{2+} binding sites (similar results have been obtained with 0.5 mM or 20 μM Ca^{2+} concentrations), in good agreement with previous kinetic studies [5,6]. It is to be noted that the difference between the T_m of the Ca^{2+}-ATPase in absence and in presence of Ca^{2+} becomes lower than 0.2°C at pH 8, therefore indicating that the stability against thermal unfolding of the E1 and E2 conformations of the Ca^{2+}-ATPase is very similar at this pH. Denaturation enthalpy of the Ca^{2+}-ATPase ranged between 250 to 300 kcal/mol of ATPase monomer, a value that is in good agreement with previous studies [7,16,18–20].

When the DSC scans are run at scanning rates lower than 30°C/h (e.g. 10°C/h) at pH 7, the endothermic peak is similar to that obtained at 30°C/h with a T_m value slightly lower (compare traces a, at 10°C/h, and b, at 30°C/h, Fig. 3). At scanning rates higher than 30°C/h the endothermic peak becomes clearly asymmetric, with the appearance of a second peak centered at higher temperatures (in the range of 55–62°C) in presence of 0.5 mM Ca^{2+} (traces c, at 60°C/h, and d, at 90°C/h, Fig. 3). The small shoulder above the main endothermic peak at high scanning rates (60 and 90°C/h) indicates kinetic segregation of the major endothermic peak into two components, and is Ca^{2+}-dependent. In fact, in presence of millimolar EGTA, at 60°C/h the endothermic peak became symmetric (data not shown). As previously described, in presence of millimolar Ca^{2+} concentrations the major endothermic peak splits into two separate peaks with $T_{m1} = 54$°C and $T_{m2} = 65$°C ([15,17], and data not shown), and the $K_{0.5}$ for the effect of Ca^{2+} is 0.8 mM (0.25 mM in [20]). The ΔH of the Ca^{2+}-dependent peak of the SR membrane thermograms is approximately 20–25% of the total ΔH of unfolding per gram of SR protein.

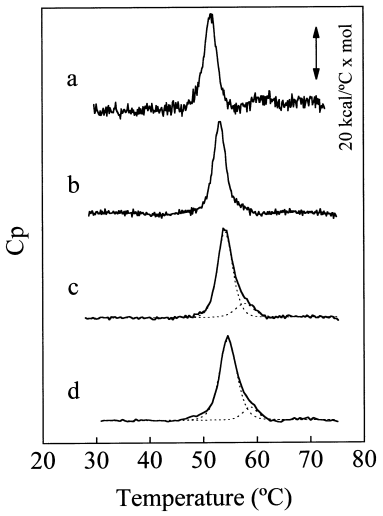

Fig. 3. Effect of scanning rate on the DSC thermograms of SR membranes in presence of 0.5 mM Ca^{2+} at pH 7. Protein concentration, 2 mg/ml. Representative DSC traces obtained at (a) 10, (b) 30, (c) 60 and (d) 90°C/h. Buffer, 20% glycerol, 0.1 M KCl and 0.02 M TES/KOH (pH 7). The results obtained by fitting traces c and d to the algorithm for a non-two-state transition (dotted lines) are also shown.

These sites are clearly not the high affinity Ca^{2+} sites of the Ca^{2+}-ATPase (with a K_d in the micromolar range for SR membranes ([34,35] and data not shown).

Trace a of Fig. 4 shows that in presence of a $C_{12}E_8$ solubilizing concentration (1 mg/ml), the thermal unfolding of SR membranes at pH 7 in presence of 5 mM Ca^{2+} and at a scan rate of 60°C/h, presents a pattern different to that seen for native SR membrane, with no appearance of the separate endothermic peak with higher T_m. Rather, a different asymmetric endothermic peak of the thermal unfolding of the Ca^{2+}-ATPase is observed and the analysis indicates that the thermogram can be deconvoluted into two peaks, implying kinetic segregation of the overall unfolding process. Purified ATPase, prepared as described in Section 2, shows DSC thermograms, in presence of 0.5 mM Ca^{2+}, of shape similar to those seen for solubilized SR membranes (Fig. 4, trace b), without segregation of a peak above the main peak, but an asymmetry of the endothermic peak, showing the trace lower slope below the T_m than above it (trace b). Noteworthy, in presence of EGTA or 5 mM Ca^{2+} the endothermic peak of the unfolding of purified ATPase became also symmetric (data not shown) with a T_m of 48.1 ± 1.1 and 53.4 ± 1°C,

respectively. The endothermic peak for purified ATPase shown in Fig. 4 (trace b) can be deconvoluted into two peaks with T_m values of 49.0 and 53.2°C, that correspond to the values of the T_m of the peaks obtained in presence of EGTA and 5 mM Ca^{2+}, respectively. This is consistent with a partial saturation of the effect of Ca^{2+} at 0.5 mM concentration. This result indicated that the different effects of millimolar Ca^{2+} concentrations on the unfolding of Ca^{2+}-ATPase, in the SR membrane and in purified ATPase, could be accounted for solubilization of the native membrane.

Saturation of the catalytic site of the Ca^{2+}-ATPase by Mg^{2+}-ATP and Mg^{2+}-ADP has been shown to strongly protect the Ca^{2+}-ATPase against denaturation by detergents [4]. Millimolar concentrations of Mg^{2+}-ATP or Mg^{2+}-ADP also stabilize the Ca^{2+}-ATPase against thermal denaturation [6,7]. To avoid problems derived from ATP hydrolysis we have studied the effect of Mg^{2+}-ADP instead of Mg^{2+}-ATP on the thermal unfolding of the Ca^{2+}-ATPase in SR membranes. The relevant thermodynamic parameters are listed in Table 1. These results show that millimolar Mg^{2+}-ADP increased the T_m at all the pH values studied with respect to the values obtained in presence of 2 mM EGTA, without significant changes of the overall unfolding enthalpy. Therefore, millimolar Mg^{2+}-ADP stabilized the Ca^{2+}-ATPase in absence of significant saturation of the high affinity Ca^{2+} sites. Noteworthy, at all pH values studied, the stabilization afforded by millimolar Mg^{2+}-ADP is stronger than that afforded by 0.5 mM Ca^{2+}, a Ca^{2+} concentration that saturates the high affinity Ca^{2+} sites of the Ca^{2+}-ATPase.

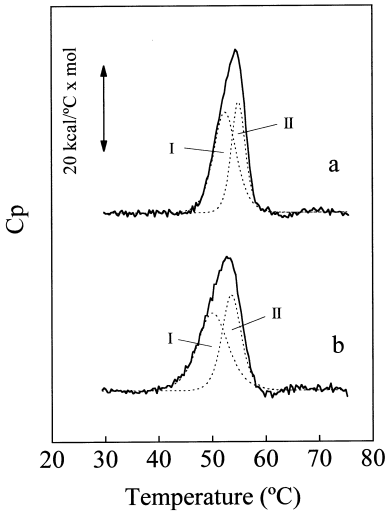

Fig. 4. Effect of detergent solubilization on the thermal denaturation pattern of SR membranes. Protein concentration, 2 mg/ml; scanning rate, 60°C/h; buffer, 20% glycerol, 0.1 M KCl, 0.02 M TES/KOH (pH 7). Trace a, SR membranes +5 mM Ca^{2+} +1 mg/ml $C_{12}E_8$; trace b, a representative DSC endotherm at 60°C/h of purified ATPase prepared by the method of MacLennan [26] in the same buffer with 0.5 mM Ca^{2+} (2 mg of protein/ml). The deconvolutions into two peaks (I and II) showed in traces a and b were obtained with the algorithm for a non-two-state transition, as indicated in Section 2. The ΔH values obtained for the two deconvoluted peaks were: trace a, 138 ± 10 (peak I) and 127 ± 11 (peak II) kcal/mol of Ca^{2+}-ATPase monomer; trace b, 134 ± 12 (peak I) and 146 ± 10 (peak II) kcal/mol of Ca^{2+}-ATPase monomer.

3.2. *Kinetic analysis of the unfolding of the Ca^{2+}-ATPase from differential scanning calorimetry data*

The thermal unfolding of a protein is a kinetic process [15]. As indicated above the unfolding of the Ca^{2+}-ATPase in the SR membrane is appropriately described with the algorithm for a non-two-state transition. Sánchez-Ruiz et al. [32] developed the theoretical background for calculation of the energy of activation for a non-two state unfolding process that conforms to the simple kinetic scheme $N \rightleftharpoons I \rightarrow D$, being N, I and D the native, intermediate and denatured protein states, respectively. As indicated in Sánchez-Ruiz et al. [32], the energy of activation of this kinetic process can be obtained from the dependence of the T_m of the thermal transitions upon the scanning rate. A plot of $\ln (\nu/T_m^2)$ versus $1/T_m$, where ν is the scanning rate (in °C/h) and the T_m is expressed in °K, results in a straight line (Fig. 5, upper panel). Since the slope of this line is $-E/R$, being E the energy of the activation of the unfolding process [32] and R the gas constant, from these plots we obtain a value for E of 132 ± 20 kcal/mol of ATPase. If the proposed kinetic scheme describes the unfolding of the Ca^{2+}-ATPase in SR membranes, this energy of activation should match the energy of activation calculated from ATPase activity measurements. The energy of activation for the denaturation process can be obtained from Arrhenius plots of the rate constants (k) of the kinetics of inactivation of the Ca^{2+}-ATPase as a function of the time of incu-

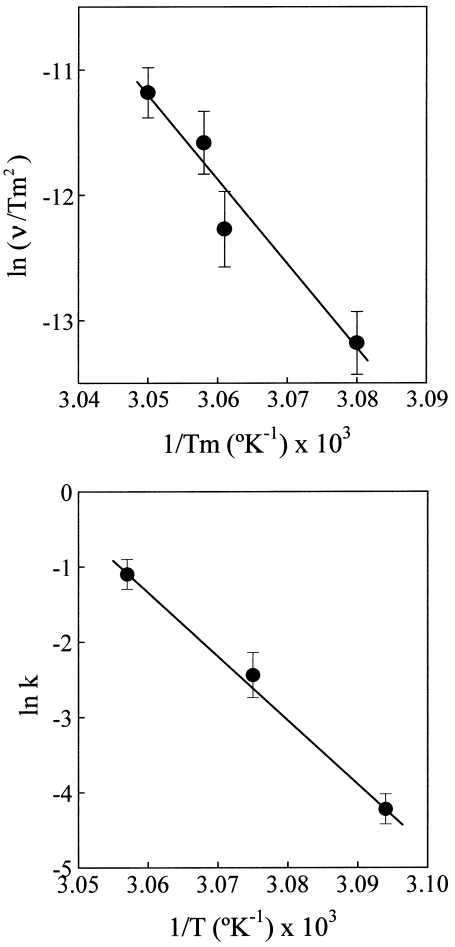

Fig. 5. Upper panel: kinetic analysis of the effect of the scanning rate on the thermal unfolding of the Ca^{2+}-ATPase in SR membranes. Plot of ln (v/T_m^2) versus $1/T_m$. Each data point was obtained from the scanning rate (v) and the T_m value of the main thermal transition at that particular scanning rate. The line was fitted by linear regression of the data, and, as indicated in Sánchez-Ruiz et al. [32], the energy of activation (E) of the process can be obtained from the slope of the line ($-E/R$). Lower panel: Arrhenius plot of the rate constant of denaturation of the Ca^{2+}-ATPase (k). k values were obtained from kinetics of denaturation of the Ca^{2+}-ATPase at different temperatures (data not shown). The dependence of the Ca^{2+}-ATPase activity of SR membranes on the time of incubation at different temperatures was done under the same experimental conditions used in DSC experiments: 20% glycerol, 0.1 M KCl, 0.5 mM Ca^{2+} and 0.02 M TES/KOH (pH 7). Protein concentration, 2 mg/ml.

bation at different temperatures in the buffer used for DSC scans (data not shown). The selected temperatures (50–54°C) overlapped with the temperature range of the thermal unfolding of the Ca^{2+}-ATPase measured in DSC experiments. From the slope of the Arrhenius plots, a value of 165 ± 20 kcal/mol of ATPase is obtained for the energy of activation of the denaturation process (Fig. 5, lower panel). This value obtained from the kinetics of inactivation of the Ca^{2+}-ATPase is close to the energy of activation derived from the kinetic analysis of the DSC thermograms collected at different scanning rates (see above). This implies a good correlation between the energy of activation of denaturation of the Ca^{2+}-ATPase in the SR membranes and the energy of activation of the major endothermic peak of DSC thermograms of SR membranes, thus strongly supporting that the unfolding process closely conforms to a kinetic scheme $N \rightleftharpoons I \rightarrow D$. In addition, this value is close to the one reported by Lepock et al. [18], 125 ± 15 kcal/mol of ATPase, the small difference being likely related to the different protocols followed to prepare SR membranes (see Section 2) and to the different composition of the buffers used for incubation of the ATPase (e.g. 20% glycerol present in our buffers, see [7]).

When the equilibrium $N \rightleftharpoons I$ is rapid in comparison with the irreversible $I \rightarrow D$ transition, an almost symmetric endothermic peak is obtained in the DSC thermograms and this type of unfolding processes can be effectively treated as a near-equilibrium process [15]. Thus, we have studied the effect of pH, EGTA, Ca^{2+} and Mg^{2+}-ADP on the van 't Hoff enthalpy (ΔH_v) of the major and almost symmetric endothermic peak of SR membranes at a scan rate of 30°C/h. ΔH_v have been calculated by using the Origin software with the non-two state algorithm fit protocol (see Section 2), and the results obtained are listed in Table 2 in the form of the apparent

Table 2
Van't Hoff enthalpy to denaturation enthalpy ratio of the thermal unfolding of the Ca^{2+}-ATPase in SR membranes

	$\Delta H_v/\Delta H$		
	0.5 mM Ca^{2+}	2 mM EGTA	2 mM EGTA/3 mM Mg^{2+}-ADP
pH 6	1.15	1.20	1.45
pH 7	1.56	1.37	1.54
pH 8	1.78	1.83	1.96

Buffers, 20% glycerol, 0.1 M KCl and 0.02 M TES/KOH (pH 7 and 8); 20% glycerol, 0.1 M KCl and 0.02 MES/KOH (pH 6); scanning rate, 30°C/h.

cooperative unit $\Delta H_v/\Delta H$. While the enthalpy of denaturation (ΔH) refers to the heat change per monomer, the van 't Hoff (ΔH_v) refers to the cooperative unit which simultaneously undergoes the thermal $N \rightleftharpoons I$ transition. The ratio $\Delta H_v/\Delta H$ is related to the minimum unit that denatures under different experimental conditions. These results yield an average cooperative unit higher than one ATPase polypeptide for the unfolding of Ca^{2+}-ATPase in SR membranes, a result which is in good agreement with the presence of oligomers of Ca^{2+}-ATPase in the SR membrane, as shown by electron microscopy image analysis [36,37] and X-ray diffraction studies of oriented SR membranes [10,11]. While Ca^{2+} had only a weak effect on the cooperativity of the unfolding process, binding of Mg^{2+}-ADP (which stabilizes the Ca^{2+}-ATPase against thermal denaturation, see above) increased the apparent size of the average cooperative unit of Ca^{2+}-ATPase unfolding in SR membranes. Noteworthy, the effect of a pH change from 6 to 8 is even stronger than the effect of Mg^{2+}-ADP on the apparent cooperative unit, as it is in the T_m. This result shows that pH changes in the range 6–8 strongly modulate the interactions between ATPase monomers in the SR membrane. The effect of the nucleotide suggests that the main contribution in these interactions came from the hydrophilic domain of the Ca^{2+}-ATPase protruding from the lipid bilayer. To further assess this point we have studied the unfolding of the Ca^{2+}-ATPase in SR membranes at a higher KCl concentration (Table 3). Similar results have been obtained with NaCl (data not shown). These results point out that, in the pH range 6–8, the cooperative unit of the unfolding ($\Delta H_v/\Delta H$) decreased to a value close to 1.0 as the KCl concentration increased. In the pH range 7–8, the increase of ionic strength produced a stabilization of the Ca^{2+}-ATPase (increase of T_m of approximately 1.0°C) similar to the stabilization afforded by Mg^{2+}-ADP, which binds to the catalytic domain located in the hydrophilic part of the Ca^{2+}-ATPase and far from the lipid–water interface [38–40]. In contrast, at pH 6, 300 mM KCl failed to produce a significant change of T_m. Therefore, these results suggest that ionic interactions make a higher contribution to the stability of the Ca^{2+}-ATPase at pH 7–8 than at pH 6.

4. Discussion

Calorimetric scans showed in this study have been obtained with essentially glycogen phosphorylase-free SR membranes, e.g. containing less than 3% (w/w) glycogen phosphorylase [22]. The complex pattern of denaturation, previously described [16,18,23], is not present in these scans, thus allowing a more accurate thermodynamic analysis of the unfolding process of the Ca^{2+}-ATPase in SR membranes.

Taking into account the similar pattern of denaturation in SR membranes and in delipidated monomeric Ca^{2+}-ATPase and the similarity of the thermodynamic parameters, T_m and ΔH (compare this work and [7]), we concluded that the delipidated mono-

Table 3
Effect of 300 mM KCl concentration on the thermodynamic parameters of the thermal unfolding of SR membrane at different pH values

	[KCl] (mM)	T_m (°C)	ΔH (kcal/mol)	$\Delta H_v/\Delta H$
pH 6	100	51.5 ± 0.2	264 ± 39	1.15
	300	51.8 ± 0.1	266 ± 25	0.96
pH 7	100	53.5 ± 0.4	267 ± 48	1.56
	300	54.4 ± 0.4	291 ± 25	1.01
pH 8	100	54.0 ± 0.1	280 ± 50	1.78
	300	55.2 ± 0.1	284 ± 23	0.92

Scanning rate, 30°C/h; buffers, 20% glycerol, 0.1 M (or 0.3 M) KCl, 0.02 M TES/KOH (pH 7 or 8), 0.5 mM Ca^{2+}; 20% glycerol, 0.1 M (or 0.3 M) KCl, 0.02 M MES/KOH (pH 6), 0.5 mM Ca^{2+}. The values of ΔH listed in the table are given in kcal/mol of Ca^{2+}-ATPase monomer, calculated as indicated in the legend of the Table 1. Values are given as mean ± S.E.M., with $n \geq 6$.

meric ATPase is structurally similar to the protein in the native membrane. This is consistent with the retention of full catalytic activity in monomeric Ca^{2+}-ATPase and also with the small perturbation of Ca^{2+} and Mg^{2+}-ATP dependence of the Ca^{2+}-ATPase activity in monomeric ATPase with respect to the Ca^{2+}-ATPase activity of SR membranes ([35] and data not shown). However, the stabilization produced by micromolar Ca^{2+} concentration (as the effect on the T_m of the unfolding process) is higher in monomeric Ca^{2+}-ATPase ($\sim$10°C at pH 7, [7]) than in native SR membranes: $\sim$1.0°C at pH 7 (this work). This result unravels that protein–protein interactions are important in the thermal stability of the Ca^{2+}-ATPase. The instability of monomeric ATPase in absence of Ca^{2+} is likely due to the instability of the transmembranal domain upon delipidation [7].

In SR membranes, Mg^{2+}-ADP and micromolar Ca^{2+} increased stability of the Ca^{2+}-ATPase against denaturation, and this is observed without splitting of the endothermic transition into two separate peaks, thus indicating a tight structural connection between catalytic and Ca^{2+} transport domain in the Ca^{2+}-ATPase. Since the catalytic site is located far away from the lipid bilayer [38–40] and the Ca^{2+} transport domain is a transmembrane domain [41,42], this implies a very compact three-dimensional structure of this ATPase. Previously, we have provided experimental evidences for a structural interconnection between both protein domains, as Mg^{2+}-ADP binding to the catalytic site protects against labeling the Ca^{2+}-ATPase by the chromophore NCD-4 at the high affinity Ca^{2+} sites [9]. DSC data also indicated that Mg^{2+}-ADP and micromolar Ca^{2+} concentrations have a synergistic effect in affording protection of Ca^{2+}-ATPase against thermal unfolding. This was noticed as well in kinetic studies of the thermal denaturation of monomeric Ca^{2+}-ATPase [6].

At high scanning rates, the pattern of thermal denaturation in presence of Ca^{2+} of the Ca^{2+}-ATPase in SR membranes is different from that at low scanning rates. In the former case, millimolar Ca^{2+} concentrations produce segregation of a transition above the main thermal transition (Fig. 3). In the latter case, no segregation of a second transition is observed. Additionally, the pattern of thermal denaturation in presence of Ca^{2+} is different in SR membranes and purified ATPase. No segregation of a second peak is seen in ATPase, but an asymmetry that is likely to be the result of a sum of the unfolding of two populations of Ca^{2+}-ATPase, corresponding to the conformations stabilized both in absence of Ca^{2+} and upon Ca^{2+} binding, respectively (Fig. 4). Moreover, the scans of SR membranes in presence of solubilizing concentrations of $C_{12}E_8$ and millimolar Ca^{2+} concentrations did not show segregation of a second peak above the main thermal transition at high scanning rates, and yield an endothermic peak of shape quite similar to that of the purified Ca^{2+}-ATPase (Fig. 4). In addition, in monomeric Ca^{2+}-ATPase is not observed segregation of a peak in presence of millimolar Ca^{2+} concentrations [7]. Taken together, these data support the hypothesis that the Ca^{2+}-dependent endothermic peak observed in SR membranes above the major endothermic peak results from protein–protein interactions in the SR membrane promoted by unspecific Ca^{2+} binding. In fact, the $K_{0.5}$ value of the effect of Ca^{2+}, 0.8 mM, on the thermal denaturation in SR membranes, is far from the $K_{0.5}$ of activation by Ca^{2+} (in the micromolar range) and close to the $K_{0.5}$ of inhibition by this cation [21]. Thus, this effect is not related to Ca^{2+} binding to the transport sites. Rather, it might be related to Ca^{2+} binding to poorly defined sites in the ATPase, which produced a thermal transition above the main transition, with a denaturation enthalpy value of 20–25% of the ΔH of the main transition. This is supported with the fact that the Ca^{2+}-binding protein calsequestrin, other major component of SR membranes, unfolds in presence of millimolar Ca^{2+} concentrations in a temperature range centered in 88°C, far from the unfolding temperature range of the ATPase (J.M. Merino and C. Gutiérrez-Merino, unpublished results). The results also show that in the SR membrane protein–protein interactions between monomers of the ATPase are enhanced in presence of millimolar Ca^{2+} concentrations, a concentration range at which inhibition of the ATPase activity by Ca^{2+} takes place [34]. A close correlation between aggregation of the Ca^{2+}-ATPase and inhibition of its activity by cationic drugs and polypeptides, such as local anesthetics and melithin, has been reported [14].

The major endothermic peak of the unfolding of the Ca^{2+}-ATPase in SR membranes can be fitted as a

non-two state unfolding process, which is scan-rate dependent. Kinetic analysis of the unfolding of the Ca^{2+}-ATPase in the SR membranes show that it can be appropriately fitted to the simple kinetic scheme:

$$N \rightleftharpoons I \rightarrow D$$

where N, I and D are native (folded), intermediate and denatured (unfolded) states, respectively. It has been shown [15] that for this kind of irreversible processes, the van 't Hoff enthalpy change (ΔH_v) can yield information regarding the apparent size of the cooperative unit for the reversible transition $N \rightleftharpoons I$. The necessary assumption is that the scanning rate is low enough to allow the unfolding to proceed near-equilibrium of the $N \rightleftharpoons I$ transition. This requirement is fulfilled for scan rates at which the endothermic peak looks highly symmetric around the T_m, as shown by the simulations reported in [15]. This is the case for DSC thermograms of SR membranes obtained at a scan rate of 30°C/h. As indicated above, the enthalpy of denaturation (ΔH) refers to the heat change per monomer and the van 't Hoff enthalpy (ΔH_v) refers to the cooperative unit which simultaneously undergoes the thermal $N \rightleftharpoons I$ transition. As a consequence, the ratio $\Delta H_v/\Delta H$ is related to the minimum unit that denatures under different experimental conditions. The ratio $\Delta H_v/\Delta H$ became higher than 1 for SR membranes, showing that the aggregation state is multimeric. This points out the existence of positive cooperativity in the thermal denaturation process of the Ca^{2+}-ATPase in SR membranes due to protein–protein interactions. Freeze-fracture studies of SR membranes reveal the existence of particles that correspond to aggregated polypeptide chains [12,43]. A recent infrared spectroscopic study of the thermal denaturation of the Ca^{2+}-ATPase has shown that this is not a two state process, and also suggest the presence of an intermediate conformation before denaturation that is associated with oligomer–monomer transitions [44,45]. Our results underlie this conclusion, and in the proposed model the intermediate state of the unfolding process in SR membranes probably reflects a distorted monomeric state of the Ca^{2+}-ATPase, as a consequence of the oligomer–monomer transition. Mg^{2+}-ADP binding increased the cooperativity in the thermal denaturation in SR membranes, indicating that Mg^{2+}-ADP binding increases the strength of interactions between Ca^{2+}-ATPase monomers in the SR membranes. This is consistent with the location of the catalytic site near the center of the oligomeric protein particles protruding from the SR membrane [40]. The strong effect of ionic strength and pH on the cooperative unit of the Ca^{2+}-ATPase unfolding in the SR membrane, points out that the hydrophilic domain of the Ca^{2+}-ATPase (containing the catalytic center), which protrudes from the lipid bilayer, plays a major role in the interactions between Ca^{2+}-ATPase monomers in the SR membrane, as suggested also by the electron density maps of SR membranes obtained from analysis of electron microscopy images [37]. It is interesting to note that high KCl concentrations produce thermal stabilization of the protein, as seen with Mg^{2+}-ADP. In contrast, this effect is accompanied by a decrease of the cooperativity, indicating that KCl produces a stabilization of the intermediate state in the unfolding process, in good agreement with previous kinetic observations [46]. In addition, the strong effect of ionic strength and pH mentioned above could explain the discrepancies in the monomer–dimer association constants previously reported by different laboratories [35,47,48].

In summary, this study of the thermal unfolding of the Ca^{2+}-ATPase shows that this protein has a compact structure, implying a tight structural interconnection between catalytic and Ca^{2+} transport domains. Cooperativity of the unfolding process is largely modulated by pH, showing a pH dependence of the extent of oligomerization in the SR membrane. The effect of Mg^{2+}-ADP and ionic strength points out that the hydrophilic part of the protein plays a major role in the interactions between ATPase monomers. Regarding structure–function relationships, these results point out that protein–protein interactions are modulated by variables that affect the catalytic activity of the protein.

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