Oligomerization of the plasma membrane calcium pump involves two regions with different thermal stability

Valeria Levi, Juan P.F.C. Rossi, Pablo R. Castello, F. Luis González Flecha*

Departamento de Química Biológica-IQUIFIB, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina

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Abstract Ca^{2+} pump dimerization was studied by using a combined approach of thermal denaturation and fluorescence resonance energy transfer. The measurement of calcium pump ability to dimerize after the unfolding of individual functional domains of the enzyme demonstrated the existence of two different regions involved in the self-association process. One of these regions is highly susceptible to thermal unfolding and was identified as the calmodulin (CaM)-binding domain. The other region whose thermal stability is higher than those of the catalytic and CaM-binding domains could be related with the previously found C28W-binding regions.

Key words: Calcium pump; Oligomerization; Thermal stability; Plasma membrane calcium pump; Calmodulin; Membrane protein

1. Introduction

Eukaryotic cells pump Ca^{2+} to the external milieu at the expense of ATP hydrolysis through the plasma membrane Ca^{2+} pump (PMCA). In erythrocytes, the main isoform of this protein is the hPMCA4b [1]. This is a single chain integral membrane protein of M_r 134 000 constituted by a large intracellular region and 8–10 transmembrane segments connected by short external loops.

The first evidence suggesting that the pump could exist in the form of dimers comes from radiation inactivation experiments on human red cell ghosts [2]. This study showed that the estimated target size of the PMCA is 251 kDa, close to twice the size of the monomeric human erythrocyte enzyme.

Later, it was demonstrated that the calcium pump isolated from human erythrocytes and reconstituted in mixed micelles of phospholipids and detergent undergoes reversible dimerization [3–7].

Vorherr et al. [8] demonstrated that the calmodulin (CaM)-binding domain of the pump is involved in the oligomerization process. Structural studies on the synthetic peptide C28W, which sequence corresponds to the above mentioned domain, showed that this fragment has a strong tendency to form aggregates and, under special conditions, to form dimers [9]. By analogy, it was proposed that this interaction could be responsible for the dimerization [8]. However, there is no experimental evidence supporting this hypothesis.

Thermal denaturation studies on the Ca^{2+} pump have demonstrated to be useful for revealing structural features of this protein [10]. Throughout this work, we employ this approach combined with fluorescence resonance energy transfer (FRET) measurements to make a close examination of the structural basis of the Ca^{2+} pump dimerization.

2. Materials and methods

2.1. Materials

All the chemicals used in this work were of analytical grade and mostly purchased from Sigma Chemical Co. (St. Louis, MO, USA). Eosin-5′-isothiocyanate (EITC) and dansyl-chloride were obtained from Molecular Probes (Eugene, OR, USA). Recently drawn human blood was obtained from the Hematology Section of the Hospital de Clínicas General José de San Martín (Argentina).

2.2. Purification of Ca^{2+} pump from human erythrocytes

CaM-depleted erythrocyte membranes were prepared as described previously [11] using 15 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS) (pH 7.4 at 4°C), 0.1 mM phenylmethyl-sulfonyl fluoride (PMSF) and 1 mM EGTA (ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) as hypotonic solution. Ca^{2+} pump was isolated by CaM affinity chromatography [12]. Fractions exhibiting the highest specific Ca^{2+}-ATPase activity were pooled. Phospholipid concentration was determined according to Chen et al. [13]. Prior to use, the enzyme (560 nM, specific Ca^{2+}-ATPase activity 11.5 μmol Pi/mg min) was kept, under liquid nitrogen, in the reconstitution buffer, containing: 300 mM KCl, 10 mM MOPS-K (pH 7.4 at 4°C), 1 mM $MgCl_2$, 2 mM EDTA, 2 mM $CaCl_2$ ($[Ca^{2+}]_{free} = 70$ μM), 2 mM 1,4-dithiothreitol, 3 mM sodium azide, 1 μM pepstatin, 10 μM leupeptin, 1 μg/ml aprotinin, 0.1 mM PMSF, 290 μM soybean phospholipids and 800 μM $C_{12}E_{10}$ (polyoxyethylene 10 lauryl ether).

2.3. Polyacrylamide gel electrophoresis (PAGE)

Integrity of purified Ca^{2+} pump was tested by SDS–PAGE. Prior to electrophoresis, samples (0.5 μg per lane) were incubated at room temperature for 5–10 min in sample buffer. SDS–PAGE (10% T and 1% C) was carried out according to the Tris/tricine method [14]. Gels were then stained with colloidal brilliant blue G [15]. Ca^{2+} pump total mass was measured by densitometric analysis using bovine seroalbumin as standard (Castello et al., personal communication).

2.4. Measurement of the Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity was measured at 37°C as the initial velocity of release of Pi from ATP, as described previously [11]. CaM-activated Ca^{2+}-ATPase activity was measured following the same procedure but adding 130 nM CaM to the medium. CaM activation was calculated as the ratio between the CaM-activated and the basal Ca^{2+}-

*Corresponding author. Fax: (54)-11-4962 5457.
E-mail: lgf@qb.ffyb.uba.ar

Abbreviations: C28W, synthetic peptide corresponding to the sequence 1086–1113 of the hPMCA4b; CaM, calmodulin; EITC, eosin-5′-isothiocyanate; FITC, fluorescein-5′-isothiocyanate; FRET, fluorescence resonance energy transfer; hPMCA4b, isoform 4b of the human plasma membrane calcium pump; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; PMCA, human plasma membrane calcium pump; PMSF, phenylmethyl-sulfonyl fluoride

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ATPase activities. The concentration of Ca^{2+} used for the activity measurement (13 μM) was determined using an Orion 9320 ion-selective Ca^{2+} electrode (Beverly, MA, USA).

2.5. Measurement of CaM-binding to the pump

CaM was dansylated as described previously [16]. Binding of dansyl-CaM to the calcium pump was determined by the FRET method described by Wrzosek et al. [17] except that the excitation wavelength was 295 nm.

2.6. Labeling of the PMCA with fluorescent probes

Ca^{2+} pump was labeled with either fluorescein-5′-isothiocyanate (FITC) or EITC as described previously [18] with some modifications. CaM-depleted erythrocyte membranes were suspended in a buffer composed of: 5 μM $CaCl_2$, 15 mM MOPS-K (pH 8.4 at 25°C), 0.1 mM PMSF, 4 mg/ml of protein and either 18 μM of FITC or EITC. These mixtures were incubated at room temperature until the complete loss of Ca^{2+}-ATPase activity (30 min for FITC and 60 min for EITC), supplemented with Tris–HCl (pH 8.4 at 25°C) up to 10 mM and washed five times with 5 μM $CaCl_2$, 15 mM MOPS-K (pH 7.4 at 10°C) and 0.1 mM PMSF. The labeled Ca^{2+} pump was purified from these membrane preparations as described in Section 2.2.

2.7. Spectroscopic measurements

The absorption spectrum of EITC-PMCA was measured in a Shimadzu UV-160A spectrophotometer using a 1 cm pathlength cuvette. The molar extinction coefficient was calculated using Tris-reacted EITC in the same buffer of EITC-PMCA samples as standard.

Fluorescence emission spectra were measured in a 3×3 mm quartz cuvette using a SLM-AMINCO BOWMAN Series 2 spectrofluorometer (Spectronic Instrument Inc., Rochester, NY, USA). PMCA and FITC-PMCA fluorescence spectra were registered with excitation at 295 nm and 495 nm, respectively. Both excitation and emission bandwidths were set at 4 nm. Appropriate blanks were subtracted from the measurements to correct background fluorescence.

The apparent efficiency of fluorescence energy transfer (E_{app}) was measured from the decrease on the donor fluorescence caused by the presence of the acceptor according to the following equation:

$$E_{app} = 1 - \frac{I_{d,a}}{I_d} \quad (1)$$

where $I_{d,a}$ and I_d are the donor emission intensity in the presence or absence of the acceptor, respectively.

The emission intensity of PMCA in the presence of dansyl-CaM was calculated as the integral of the PMCA peak, since the donor and acceptor emission spectra are not overlapped.

The emission spectra of the system composed of FITC-PMCA and EITC-PMCA, which spectra are partially overlapped, can be considered a linear combination of the emission spectra of each species as:

$$I(\lambda) = \sum_j \omega_j \cdot I^\circ_j(\lambda) \quad (2)$$

where $I(\lambda)$ is the emission intensity of the mixture, $I^\circ_j(\lambda)$ is the emission intensity spectrum of the isolated j component and ω_j is the weight factor of each component in the mixture.

Deconvolution of the spectrum of each mixture in the individual FITC-PMCA and EITC-PMCA spectra was done by fitting Eq. 2.

The FITC-PMCA fluorescence intensity in a given mixture was calculated as:

$$I_{\text{FITC-PMCA}} = \int \omega_{\text{FITC-PMCA}} \cdot I^\circ{}_{\text{FITC-PMCA}}(\lambda)\,d\lambda \cong \omega_{\text{FITC-PMCA}} \sum_\lambda I^\circ{}_{\text{FITC-PMCA}}(\lambda) \cdot \Delta\lambda \quad (3)$$

The distance R_o at which the probabilities of spontaneous donor fluorescence and energy transfer are equal to each other was calculated according to Förster [19]:

$$R_o = 9.79 \cdot 10^3 \cdot \left(\frac{\kappa^2 \cdot \phi_{\text{FITC-PMCA}} \cdot J}{\eta^4} \right)^{1/6} \text{(in Å)} \quad (4)$$

where η is the refractive index of the medium, κ^2 is the orientation factor between the transition moments of the donor and acceptor, $\phi_{\text{FITC-PMCA}}$ is the fluorescence quantum yield of FITC bound to the Ca^{2+} pump and J is the spectral overlap integral defined by:

$$J = \int I_{\text{FITC-PMCA}}(\lambda) \cdot \varepsilon_{\text{EITC-PMCA}}(\lambda) \cdot \lambda^4 d\lambda \ \text{(in cm}^3\ \text{M}^{-1}\text{)} \quad (5)$$

where λ is the wavelength, $I_{\text{FITC-PMCA}}(\lambda)$ is the fluorescence of the donor with the total intensity ($I_{\text{FITC-PMCA}}$) normalized to unity, and $\varepsilon_{\text{EITC-PMCA}}(\lambda)$ is the molar extinction coefficient of the acceptor, both at λ.

The fluorescence quantum yield of FITC bound to the Ca^{2+} pump was calculated at 25°C as described previously [20] according to:

$$\phi_{\text{FITC-PMCA}} = \frac{I_{\text{FITC-PMCA}} \cdot \phi_{\text{reference}} \cdot A_{\text{reference}}}{I_{\text{reference}} \cdot A_{\text{FITC-PMCA}}} \quad (6)$$

where A is the absorbance and I is the total emission intensity, both at the excitation wavelength. These quantities were measured for the sample and a reference of known quantum yield, e.g. quinine in 1 N H_2SO_4 ($\phi_{\text{quinine}} = 0.55$).

2.8. Data analysis

Data presented in this work included at least two independent experiments. The equations were fitted to the experimental data using a non-linear regression procedure based on the Gauss–Newton algorithm [21]. The dependent variable was assumed homoscedastic (constant variance), and the independent variable was considered to have negligible error.

3. Results and discussion

3.1. Monitoring PMCA dimerization by using FRET

FRET is a standard method for the study of interactions among molecules [20]. It was mainly employed to measure relative molecular distances [22] and less frequently to determine kinetic and thermodynamic properties of molecular processes [23,24]. Particularly, protein dimerization can be measured by using two populations of the protein, each one labeled with a fluorophore, constituting a FRET donor–acceptor pair [25,26].

Under the controlled labeling conditions used in this work, the fluorophore FITC binds to the PMCA reacting only with lysine 591 of the hPMCA4b isoform [27]. Structural similarities between FITC and EITC suggest that EITC could be attached to the same site, as was observed for the Ca^{+2} pump of sarcoplasmic reticulum [18]. This binding site is close

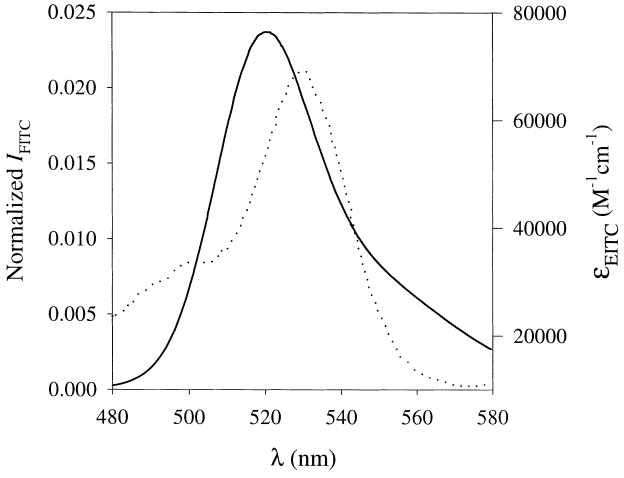

Fig. 1. Overlap between the emission of FITC-PMCA and the absorption of EITC-PMCA. The normalized emission spectrum of FITC-PMCA (continuous line) and the absorption spectrum of EITC-PMCA (dotted line) were measured as described in Section 2.

to the location of the adenosine moiety in the ATP bound enzyme [28]. Fig. 1 shows that this donor–acceptor pair has the advantage of the high extinction coefficient of the acceptor and an excellent degree of overlap between the emission spectrum of the donor (FITC) and the absorption spectrum of the acceptor (EITC). The overlap integral J was calculated as described in Section 2 giving a value of $3.47 \cdot 10^{-15}$ cm^3 M^{-1}. The quantum yield of FITC bound to the Ca^{2+}-ATPase was 0.13. The orientation factor κ^2 was considered to be 2/3; according to Lakowicz [29], this approximation introduces a minor error in R_o. Taking into account these values and considering 1.33 for the refractive index, the calculated Förster distance R_o was 50.3 Å. Since the energy transfer efficiency depends on the sixth power of the donor–acceptor distance [19], EITC-PMCA molecules could only be excited if they were at a distance in the order of R_o with respect to FITC-PMCA molecules.

Fig. 2A shows the fluorescence emission spectra of FITC-PMCA and EITC-PMCA (continuous lines). The numerical addition of these individual spectra gives the composed spectrum represented as a continuous line in Fig. 2B. In this figure, the spectrum obtained after mixing the donor and the acceptor labeled pump is also included (dashed line). This spectrum was deconvoluted as described in Section 2, obtaining the spectra represented by dashed lines in Fig. 2A. A decrease of the fluorescence intensity corresponding to the FITC peak can be observed concomitantly with an increase of the intensity associated to the EITC peak, indicating the existence of energy transfer between the probes. The time required to reach the steady state, determined by following the time course of energy transfer (not shown), was 70 min. Because the R_o value is in the order of the molecular size of P-ATPases [28], the existence of energy transfer implies the association between Ca^{2+} pump molecules. Therefore, the Ca^{2+} pump dimer assembly could be accurately monitored by FRET between FITC-PMCA and EITC-PMCA.

The energy transfer efficiency measured in an equimolar mixture of FITC-PMCA and EITC-PMCA (total enzyme concentration 440 nM) was 0.12, suggesting that dimerization events are not frequent or that the distances between probes within the dimer are quite large. The high dissociation constant of PMCA dimers measured under identical experimental conditions (unpublished results) supports the first hypothesis.

On the other hand, SDS–PAGE analysis of the enzyme (Table 1) revealed a single band corresponding to the hPMCA4b monomer size, indicating that SDS probably dissociates the dimers. It can be concluded that the interactions stabilizing PMCA dimers are not as strong as those involved in other membrane protein dimers as glycophorin A [26], which dimerization could be monitored by SDS–PAGE. Thus, the FRET method constitutes an accurate choice to study weak-interacting oligomers.

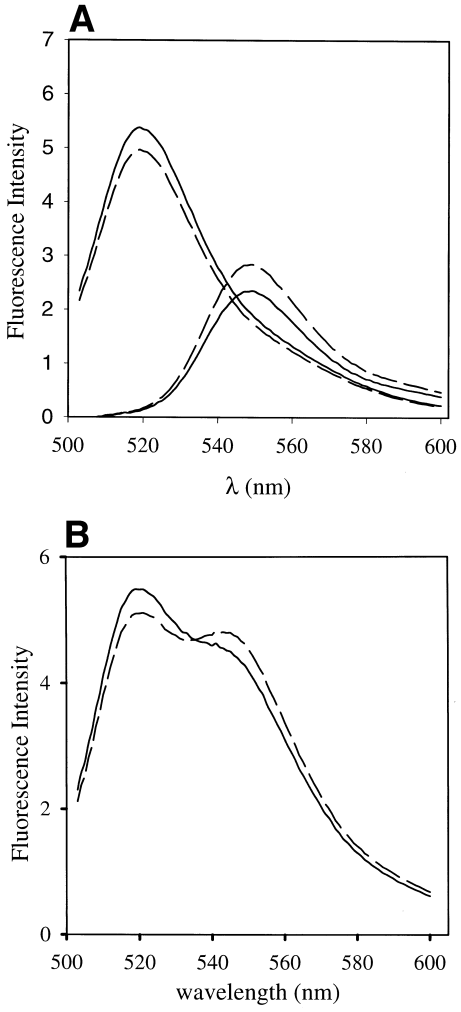

Fig. 2. Energy transfer between FITC-PMCA and EITC-PMCA. (A) Emission spectra of 220 nM FITC-PMCA and 220 nM EITC-PMCA (continuous line) were measured between 500 and 600 nm. The dashed lines correspond to the spectra obtained by deconvolution of the spectrum measured after mixing for 1.5 h FITC-PMCA and EITC-PMCA up to the concentrations previously mentioned. The peaks located at 520 nm and 560 nm correspond to FITC and EITC fluorescence emission, respectively. (B) Emission spectra obtained by numerical addition of the individual species spectra (continuous line) and after mixing during 1.5 h both species (dashed line).

3.2. Selective unfolding of specific domains of the Ca^{2+} pump

Protein association requires specific regions on each of the monomers, so it can occur only if these regions are correctly folded. Under denaturing conditions, the structural domains of a protein usually unfold independently in an all-or-none process [30].

Table 1
Thermal denaturation of the Ca^{2+} pump

Denaturation condition	Ca^{2+}-ATPase activity		Binding to dansyl-CaM (FRET efficiency)	SDS–PAGE analysis
	basal	CaM-activated		
Native	100 ± 8	159 ± 17	0.15 ± 0.01	Single band at M_r 134 000
1.5 h at 44°C	52 ± 3	44 ± 8	0	Single band at M_r 134 000
7 h at 44°C	6 ± 3	ND	0	Single band at M_r 134 000
10 min at 100°C	0	ND	ND	Higher M_r aggregates

ND = non-determined.

To determine the denaturing conditions in which different functional domains of the PMCA are unfolded, the denaturation process of the calcium pump was followed by measuring: (a) Ca^{2+}-ATPase activity in the presence or absence of CaM; (b) binding of dansyl-CaM; and (c) SDS–PAGE analysis.

Thermal denaturation was performed by incubating Ca^{2+} pump solubilized in reconstitution buffer at 44°C. In a previous paper, we showed that under these conditions, thermal inactivation takes place through a common mechanism in the range 33–45°C that is well described by an exponential function with a lifetime of 2.5 h at 44°C [10].

Binding to dansyl-CaM was assayed by measuring the energy transfer efficiency between the Trp residues of the Ca^{2+} pump and dansyl attached to the CaM. Inset to Fig. 3 shows that addition of dansyl-CaM produced a decrease on Ca^{2+} pump intrinsic fluorescence, indicating that the enzyme and dansyl-CaM constitute a good energy transfer pair. Considering that R_o for this donor–acceptor pair is 21 Å [20] and the high affinity of CaM for the PMCA [3], the energy transfer efficiency measured (Table 1) indicates that the average distance between dansyl attached to CaM and the Trp residues of the pump is 28 Å (see [29] for details of this calculation).

Fig. 3 shows the binding of dansyl-CaM and CaM activation during the thermal denaturation of the Ca^{2+} pump. Energy transfer efficiency decreased with a lifetime of approximately 15 min concomitantly to CaM activation, suggesting that both processes are related.

Table 1 shows that after 1.5 h of incubation at 44°C, both binding to dansyl-CaM and CaM activation are completely lost, but the remaining Ca^{2+}-ATPase activity was 52%. After 7 h of incubation at 44°C, the remaining activity of the enzyme was 6% and no binding to dansyl-CaM nor activation by CaM were observed.

SDS–PAGE analysis revealed that inactivation at 44°C did not produce aggregation or degradation of the Ca^{2+} pump. In contrast, after 10 min of incubation at 100°C, the enzyme lost its activity but, in this case, the enzyme undergoes irreversible aggregation.

These results suggest that at early stages of thermal denaturation at 44°C, the CaM-binding domain is non-functional while the catalytic domain is only partially inactivated. At longer times of denaturation, also the catalytic domain is denatured.

3.3. Mapping the dimerization domain of the PMCA

A convenient strategy to determine the regions of the pump involved in the dimerization consists in measuring the ability of the Ca^{2+} pump to dimerize after unfolding different domains of this protein.

Fluorescence anisotropy measurement has shown that the time course of the thermal denaturation of the labeled enzyme

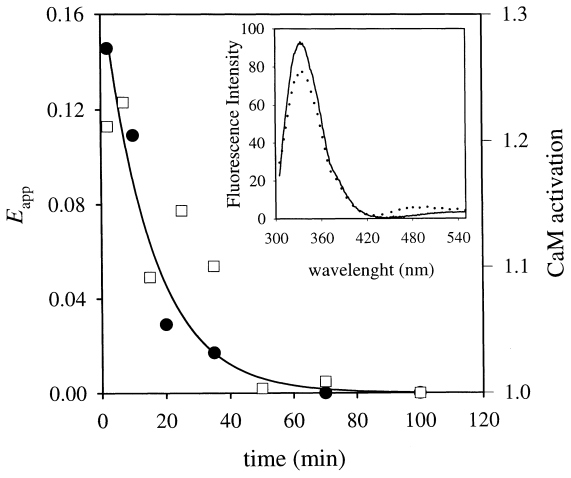

Fig. 3. Kinetics of thermal unfolding of the CaM-binding domain. The enzyme (400 nM) was incubated at 44°C in the conditions indicated in the text. After different times, aliquots were taken to test energy transfer with dansyl-CaM (●) and CaM activation (□), as described in Materials and Methods. Inset: Energy transfer between PMCA and dansyl-CaM. The emission spectra of 160 nM PMCA were registered in the presence of 500 nM of CaM (continuous line) or dansyl-CaM (dotted line), as described in Section 2. The peaks at 333 and 480 nm correspond to the PMCA and dansyl-CaM, respectively.

is identical to the corresponding non-labeled enzyme followed by measuring the Ca^{2+}-ATPase activity (unpublished results). Therefore, the selective unfolding of the labeled pump was done using the conditions established in Section 3.2, and the dimerization in each condition was evaluated measuring the apparent FRET efficiency between FITC-PMCA and EITC-PMCA (Table 2).

When native FITC-PMCA was mixed with EITC-PMCA denatured 7 h at 44°C, we verified the existence of energy transfer. On the contrary, when native FITC-PMCA was mixed with EITC-PMCA denatured 10 min at 100°C, no energy transfer was detected. This result indicates that the structure of a protein region involved in the oligomerization is preserved after 7 h of thermal denaturation at 44°C, but it is damaged when heating under stronger conditions.

It has been proposed that the CaM-binding domain mediates the self-association of the PMCA [8]. According to the results shown in Fig. 3 and Table 1, the CaM-binding domain lost its functionality after 1.5 h of incubation at 44°C. This profile does not correspond to the one observed for the dimerization region described above. Two possible explanations could account for this discrepancy: (a) the non-functional CaM-binding domain is still able to participate in the dimerization process; or (b) the dimerization of the Ca^{2+} pump involves another region of the PMCA.

Table 2
Energy transfer between FITC-PMCA and EITC-PMCA under different stages of thermal denaturation

FITC-PMCA	EITC-PMCA	Addition	FRET efficiency
Native	Native	None	0.12 ± 0.01
Native	Denatured 7 h at 44°C	None	0.12 ± 0.01
Native	Denatured 10 min at 100°C.	None	0
Denatured 1.5 h at 44°C	Denatured 1.5 h at 44°C	None	0
Native	Native	130 nM CaM	0.09 ± 0.01
Native	Native	6 μM CaM	0

To test which of these hypotheses is correct, we assayed the energy transfer between FITC-PMCA and EITC-PMCA both incubated in separated tubes during 1.5 h at 44°C. We verified no energy transfer indicating that there is no association when the CaM-binding domain is non-functional. This result indicates that: (a) the region of the pump involved in the dimerization and preserved after 7 h of denaturation at 44°C does not correspond to the CaM-binding domain and; (b) the association only occurs if at least the CaM-binding domain of one of the monomers has its native structure preserved. This last statement was confirmed by the progressive loss of energy transfer after the addition of increasing CaM concentrations to the native enzyme (Table 2), agreeing with Vorherr et al. [8] who showed that CaM tends to maintain the enzyme in the monomeric state hindering its oligomerization.

On the other hand, it was demonstrated that the dimeric calcium pump is able to bind one CaM molecule [8]. In that paper, it was suggested that this result could be compatible with either the binding of the CaM molecule to only one monomer of the dimeric pump, or with its simultaneous binding to both monomers. However, at that time, no decision between these two alternatives could be made. According to the results presented in this work, one of the CaM-binding domains remains free after dimerization and thus, it can bind CaM.

3.4. Concluding remarks

The combined strategy of thermal denaturation and FRET measurements used in this work allowed to demonstrate the existence of two different regions of the PMCA involved in the dimerization. One of these regions is highly susceptible to thermal unfolding and could be identified with the CaM-binding domain, as was previously proposed. The structure of the other region was preserved even if the enzyme was completely inactivated; therefore, this region is probably not included within the catalytic or CaM-binding domains since they are unfolded after total thermal inactivation.

It was demonstrated that C28W peptide, which sequence corresponds to the CaM-binding domain of the calcium pump, interacts with two different regions of the enzyme molecule [31]. The first is a short segment (residues 537–544) located in the second cytoplasmic loop just between the FITC-binding site (Lys 591) and the phosphorylation site (Asp 465). The second region (residues 206–271) is located in the cytoplasmic loop between the second and the third putative transmembrane segments. These authors proposed that the interaction of the CaM-binding domain with both regions located in the same protein molecule produces the auto-inhibition of the pump which is released by CaM-binding. We could rather speculate that the dimer formation occurs via the interaction of the CaM-binding domain of one pump molecule with at least one of the previously mentioned segments of another pump molecule.

The functional significance of PMCA dimerization is a controversial point. Kosk-Kosicka and Bzdega [3] found an increase of Ca^{2+}-ATPase activity at high enzyme concentrations that was attributed to oligomerization. However, recent findings of Bredeston and Rega [32] cast doubt on this causal relation. Our results shows that dimers could be formed by a native and an inactive monomer of the enzyme, suggesting that the dimerization itself does not correlate with, or has minimal effects on the function of the Ca^{2+} pump.

The study of the thermal stability of the calcium pump structural domains, currently in progress in our laboratory, will contribute to the further comprehension of the dimerization mechanism and its importance for the enzyme functionality.

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