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Fluorescence Studies on Calmodulin Binding to Erythrocyte Ca^{2+}-ATPase in Different Oligomerization States†

Danuta Kosk-Kosicka,*,‡ Tomasz Bzdega,‡ and J. David Johnson§

Department of Biological Chemistry, University of Maryland, School of Medicine, 660 West Redwood Street, Baltimore, Maryland 21201, and Department of Physiological Chemistry, The Ohio State University, Medical Center, Columbus, Ohio 43210

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ABSTRACT: The fluorescent spinach calmodulin derivative 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid-calmodulin (MIANS–CaM) was used to investigate calmodulin interaction with the purified, detergent-solubilized erythrocyte Ca^{2+}-ATPase. Previous studies have shown that the Ca^{2+}-ATPase exists in equilibria between monomeric and oligomeric forms. We report here that MIANS–CaM binds to both enzyme forms in a Ca^{2+}-dependent manner, with a ~50% fluorescence enhancement. These findings confirm our previous observation that enzyme oligomers retain their ability to bind calmodulin, even though they are fully activated in the absence of calmodulin. The Ca^{2+} dependence of MIANS–CaM binding to monomeric Ca^{2+}-ATPase is of higher affinity ($K_{1/2}$ = 0.09 μM Ca^{2+}) and less cooperative (n_H = 1.1) than the Ca^{2+} dependence of enzyme activation by MIANS–CaM ($K_{1/2}$ = 0.26 μM Ca^{2+}, n_H = 2.8). These Ca^{2+} dependences and the order of events, in which calmodulin binding precedes enzyme activation, demonstrate that calmodulin indeed could be a physiological activator of the monomeric enzyme. The calcium dependence of calmodulin binding to oligomeric Ca^{2+}-ATPase occurs at even lower levels of Ca^{2+} ($K_{1/2}$ = 0.04 μM Ca^{2+}), in a highly cooperative fashion (n_H = 2.3), and essentially in parallel with enzyme activation ($K_{1/2}$ = 0.05 μM Ca^{2+}, n_H = 2.9). The observed differences between monomers and oligomers suggest that the oligomerized Ca^{2+}-ATPase is in a conformation neccessary for efficient, cooperative calcium binding at nanomolar Ca^{2+}, which the monomeric enzyme acquires only upon interaction with calmodulin. Fluorescence titrations of Ca^{2+}-ATPase oligomers and monomers with MIANS–CaM reveal significant difference, indicating that the oligomer is saturated near 0.5 mol of calmodulin/mol of Ca^{2+}-ATPase while the monomer is saturated near 1 mol of calmodulin/mol of Ca^{2+}-ATPase. These results suggest the possibility that oligomerization of the Ca^{2+}-ATPase monomers results in the elimination of one calmodulin binding site per every two associated enzyme molecules.

Calmodulin has been shown to modulate the activity of a broad variety of enzymes, including the Ca^{2+}-ATPase of plasma membranes (Manalan & Klee, 1984; Schatzmann, 1982; Johnson & Mills, 1986). Despite extensive studies, the exact mechanism of calmodulin action on many enzymes has not been resolved. We have approached this problem by means of comparative studies of calmodulin's interaction with two forms of the same enzyme, the purified erythrocyte Ca^{2+}-ATPase, that differ in their oligomerization state. Previously we have studied the binding of calmodulin to the Ca^{2+}-ATPase by means of calmodulin affinity chromatography (Kosk-Kosicka & Bzdega, 1988). We have shown that both enzyme forms bind calmodulin whereupon only the interaction between calmodulin and the monomeric enzyme results in Ca^{2+}-ATPase activation. From the calmodulin dependence of the Ca^{2+}-ATPase activity of the monomeric form, we have derived a molar stoichiometry of 0.73:1 of calmodulin to enzyme, which implies that one calmodulin molecule binds to one enzyme monomer for activation. The stoichiometry of calmodulin binding to the oligomeric enzyme could not be measured by activity because the oligomers are fully active in the absence of calmodulin, and binding of calmodulin does not affect their Ca^{2+}-ATPase activity.

In the present study, we use spinach calmodulin, labeled at cysteine-26 with a sulfhydryl-selective fluorescent probe which undergoes a fluorescence increase with target protein binding (Mills et al., 1988). This 2-(4-maleimidoanilino)-naphthalene-6-sulfonic acid–calmodulin (MIANS–CaM)[1] was

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* To whom correspondence should be addressed.
‡ University of Maryland.
§ The Ohio State University.

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used previously to characterize calmodulin binding to myosin light chain kinase, calcineurin, and caldesmon by monitoring the fluorescence enhancement that occurs when it interacts with target proteins (Mills et al., 1988). In this paper, we quantify the calcium-dependent binding of MIANS–CaM to the monomers and oligomers of the Ca^{2+}-ATPase in order to establish the relationship between calmodulin binding and the regulation of enzyme activity in both enzyme forms.

Materials and Methods

Egg yolk phosphatidylcholine (P5763) and CNBr-activated Sepharose 4B were purchased from Sigma, $C_{12}E_8$ was from Nikkol, Japan. 2-(4-Maleimidoanilino)naphthalene-6-sulfonic acid (MIANS) was obtained from Molecular Probes. Spinach calmodulin was purified and subsequently labeled with MIANS as described previously (Mills et al., 1988). The stoichiometry of labeling was 1:1. Calmodulin isolated from bovine testes was a generous gift of Professor Robert Steiner of the University of Maryland Baltimore campus. Coupling of bovine calmodulin to Sepharose was performed in accordance with Pharmacia instructions, as described earlier (Kosk-Kosicka & Bzdega, 1988).

Purification of Ca^{2+}-ATPase. The Ca^{2+}-ATPase was purified from erythrocyte ghosts by calmodulin affinity column chromatography in the presence of the nonionic detergent $C_{12}E_8$ as described previously (Kosk-Kosicka & Inesi, 1985; Kosk-Kosicka et al., 1986). The enzyme was stored at −80 °C in elution buffer containing 10 mM Tris–maleate, pH 7.4, 130 mM KCl, 0.5 mM $MgCl_2$, 5 mM EGTA, 20% glycerol, 750 μM $C_{12}E_8$, 2 mM DTT, and 0.02% of a sonicated suspension of egg yolk phosphatidylcholine.

Protein Assay. The concentration of enzyme protein in the eluate was measured by the Lowry et al. (1951) method modified according to Bensadoun and Weinstein (1976) as well as by the Bio-Rad Protein Micro-assay, based on the Bradford dye-binding procedure (Bradford, 1976). Both methods give values within 5% error. Bovine serum albumin was used as a standard. Spinach calmodulin concentration was determined by the method of Bradford with native bovine brain calmodulin as the standard.

As the complete primary structure of the erythrocyte Ca^{2+}-ATPase has not been elucidated yet, the molecular weight of 140000 determined by gel electrophoresis was used in calculations of the stoichiometry of calmodulin binding to the enzyme (Kosk-Kosicka et al., 1986). The molecular weight of the enzyme could be within 5% error [if the molecular weight ranges from 135000 to 142000; see Verma et al., (1988)]. For spinach calmodulin, the molecular weight of 16684 based on its amino acid sequence was used (Lucas et al., 1984).

Ca^{2+}-ATPase Activity. Ca^{2+}-ATPase activity was determined by measurement of inorganic phosphate production, generally as described previously (Kosk-Kosicka & Bzdega, 1988). The assay was performed in the reaction mixture containing 50 mM Tris–maleate, pH 7.5, 130 mM KCl, 8 mM $MgCl_2$, 3 mM ATP, 1 mM EGTA, and $CaCl_2$ in concentrations yielding the required free [Ca^{2+}]. The reaction volume was either 50 or 100 μL. Appropriate aliquots of the Ca^{2+}-ATPase in the elution buffer were added to achieve the desired enzyme concentration. The concentration of $C_{12}E_8$ was kept constant at 150 μM. The amount of added calmodulin is given in the figures, accordingly. The reaction was started with 3 mM ATP and carried out for up to 30 min at 37 °C. Depending on the enzyme concentration, various aliquots were withdrawn for subsequent colorimetric inorganic phosphate measurement with malachite green (Lanzetta et al., 1979). Steady-state velocities were obtained from plots of inorganic phosphate production which were linear with time.

Calmodulin Binding. The interaction of MIANS–CaM with the Ca^{2+}-ATPase was determined by measurement of the fluorescence intensity. Excitation and emission wavelengths were 320 and 440 nm, respectively. The enzyme at a constant concentration, 12 or 60 nM, in a reaction mixture identical with that used for the Ca^{2+}-ATPase activity assay was titrated either with MIANS–CaM or with Ca^{2+} in the presence of 100 nM MIANS–CaM. From MIANS–CaM titrations of the Ca^{2+}-ATPase, MIANS–CaM titrations of buffer alone were subtracted. The buffer system did not interfere with MIANS–CaM ability to specifically report the binding of the Ca^{2+}-ATPase to this fluorescent biologically active calmodulin. All measurements were carried out at room temperature in a Perkin-Elmer LS5 spectrofluorometer or an SLM 8000 fluorometer.

The data obtained in studies on the binding of the MIANS–CaM to the Ca^{2+}-ATPase were analyzed according to eq 1 (Richards & Vithayathil, 1959) where r = the molar ratio

$$\% F = 50\left[r + 1 + K' - \sqrt{(r + 1 + K')^2 - 4r}\right] \quad (1)$$

of CaM/enzyme, $\% F$ = the percent of the total fluorescence change, which is directly proportional to the concentration of the enzyme–CaM complex, and K' is the dissociation constant/total concentration of the enzyme.

Fitting of the experimental points representing the dependence of Ca^{2+}-ATPase activity on calmodulin concentration (in Figure 1) and fluorescence and Ca^{2+}-ATPase activity on enzyme concentration (in Figure 3A,B) was obtained by using an iterative nonlinear regression method (Koeppe & Hamann, 1980).

Because of signal to noise limitations, our MIANS–CaM titrations could not be performed on enzyme concentrations below 12 nM. At this concentration, the enzyme is predicted to be essentially 60% monomer and 40% oligomer, assuming a $K_{1/2}$ = 15 nM for the monomer–oligomer transformation. This could explain the fact that our monomer data (Figure 4A) deviate from the theoretical curve.

Free Calcium. Free Ca^{2+} concentrations were calculated (Fabiato & Fabiato, 1979) from total calcium and total EGTA, based on the constants given by Schwartzenbach et al. (1957) and the pK values given for EGTA by Blinks et al. (1982), taking into account pH, ionic strength, and magnesium and ATP concentrations. Total calcium was measured by atomic absorption.

Results

Stimulation of Ca^{2+}-ATPase Activity by Native and Modified Spinach Calmodulins. The stimulation of the Ca^{2+}-ATPase activity caused by the native and modified (MIANS–CaM) spinach calmodulins was compared to that of bovine calmodulin. As shown in Figure 1, all these calmodulins stimulated Ca^{2+}-ATPase activity of the predominantly monomeric, 12 nM enzyme, whereas none of them stimulated activity of the oligomeric form. Half-maximal stimulation was observed at very similar concentrations, 5–7 nM, for all three calmodulins. In all cases, maximal stimulation was reached upon addition of around 20 nM calmodulin, when the molar ratio of calmodulin to the enzyme was around 2:1. As we have shown previously for the bovine calmodulin

[1] Abbreviations: $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; MIANS–CaM, 2-(4-maleimidoanilino)-naphthalene-6-sulfonic acid–calmodulin.

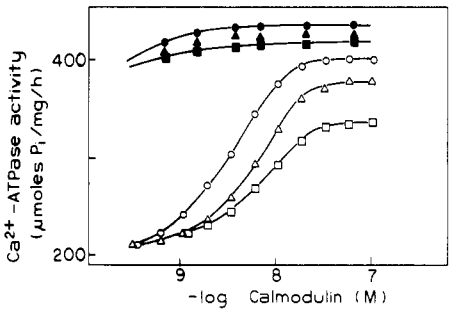

FIGURE 1: Stimulation of Ca^{2+}-ATPase activity by bovine (○), spinach (△), and MIANS–spinach (□) calmodulins. The reaction mixture contained 50 mM Tris–maleate, pH 7.5, 130 mM KCl, 150 μM $C_{12}E_8$, 8 mM $MgCl_2$, 1.0 mM EGTA, and sufficient $CaCl_2$ (1.02 mM) to yield 17.75 μM free Ca^{2+} and 3 mM ATP. [Ca^{2+}-ATPase] was 12 nM in the assay for the predominantly monomeric enzyme (open symbols) and 60 nM for the oligomers (closed symbols). Calmodulins were added to a final concentration of up to 200 nM. The reaction was performed as described under Materials and Methods.

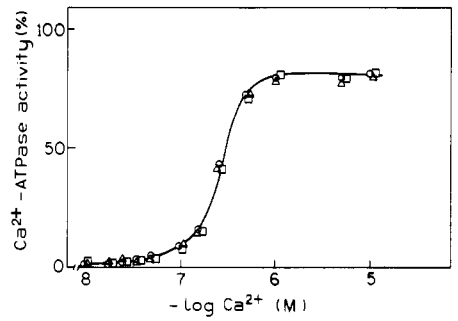

FIGURE 2: Calcium concentration dependence of stimulation of monomeric Ca^{2+}-ATPase activity by 100 nM bovine (○), spinach (△), and MIANS–spinach (□) calmodulin. The Ca^{2+}-ATPase activity was measured as described under Materials and Methods and in Figure 1. Various amounts of $CaCl_2$ were added to obtain the calcium concentration specified in the horizontal axis. The specific activity equal to 100% for each calmodulin was as shown in Figure 1, respectively.

at these conditions, the stoichiometry of binding for activation is 1:1 (Kosk-Kosicka & Bzdega, 1988). The maximal level of stimulation by the derivatized calmodulin was somewhat lower, by about 20%, than that of the native spinach calmodulin, which itself stimulated 15% less than the bovine calmodulin.

Figure 2 shows the Ca^{2+} concentration dependence of 100 nM bovine CaM, spinach CaM, and MIANS–CaM activation of monomeric Ca^{2+}-ATPase to be identical. In each case, calcium half-maximally activated the monomeric enzyme at 0.25 μM Ca^{2+} in a cooperative fashion (n_H = 2.7). The results in Figures 1 and 2 demonstrate that the MIANS–CaM has similar properties to the bovine calmodulin with respect to the stimulation of the Ca^{2+}-ATPase activity. Thus, the results of the present study with spinach CaM could be related to our previous reports using bovine CaM (Kosk-Kosicka & Bzdega, 1988; Kosk-Kosicka & Inesi, 1985; Kosk-Kosicka et al., 1986).

Ca^{2+} Dependence of Calmodulin Binding and Activation of Ca^{2+}-ATPase. MIANS–CaM was used to compare the calcium dependence of calmodulin binding to the enzyme with the calcium dependence of its stimulation of Ca^{2+}-ATPase activity. MIANS–CaM binds to both enzyme forms, with a ~50% fluorescence enhancement (Figure 3A, inset), confirming our previous finding that the oligomerized enzyme has not lost its ability to interact with calmodulin (Kosk-Kosicka & Bzdega, 1988).

The Ca^{2+} dependence of calmodulin binding and stimulating Ca^{2+}-ATPase activity is shown in Figure 3A for the predominantly monomeric enzyme and in Figure 3B for the oligomeric

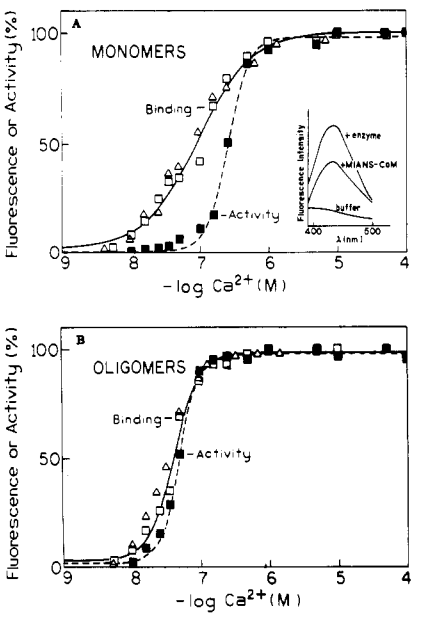

FIGURE 3: [Ca^{2+}] dependence of calmodulin binding and activation of monomeric and oligomeric Ca^{2+}-ATPase. Calmodulin binding (open symbols) and Ca^{2+}-ATPase activity (closed symbols) of the predominantly monomeric, 12 nM (A), and oligomeric, 60 nM (B), enzyme were studied. Ca^{2+}-ATPase activity (■) was measured as described under Materials and Methods and in Figure 1. Binding of calmodulin was measured by the enhancement of MIANS–CaM fluorescence intensity, as described under Materials and Methods, either in the presence of ATP (□) or in its absence (△). The reaction mixture was as in the Ca^{2+}-ATPase activity assay, except [$MgCl_2$] was 5 mM when no ATP was added. 100 nM MIANS–CaM was used in all assays. 100% fluorescence change is equivalent to a 48% and 53% increase of MIANS–CaM fluorescence intensity upon its reaction with enzyme monomers and oligomers, respectively, as measured at saturating Ca^{2+} concentrations. Calcium titrations of MIANS–CaM in buffer alone produced no significant fluorescence increase. The inset shows fluorescence emission spectra of buffer alone, buffer plus 100 nM MIANS–CaM, and 100 nM MIANS–CaM plus 60 nM Ca^{2+}-ATPase at 17.75 μM free calcium.

Table I: Comparison of the [Ca^{2+}] Dependence of Calmodulin Interaction with Monomeric and Oligomeric Enzyme and Their Ca^{2+}-ATPase Activity[a]

function measured	prevailing enzyme form			
	monomeric (12 nM)		oligomeric (60 nM)	
	K_d (pCa)	n_H	K_d (pCa)	n_H
CaM binding	7.04 ± 0.04	1.12 ± 0.10	7.38 ± 0.02	2.30 ± 0.24
Ca^{2+}-ATPase act. with CaM	6.59 ± 0.02	2.77 ± 0.25	7.31 ± 0.01	2.96 ± 0.18
Ca^{2+}-ATPase act. without CaM	6.25 ± 0.10	0.85 ± 0.18	7.15 ± 0.01	3.09 ± 0.09

[a] Experimental conditions are described under Materials and Methods and in the Legend to Figure 3.

enzyme. The monomeric enzyme displays different Ca^{2+} dependencies for the two reactions (Figure 3A and Table I). The interaction of calmodulin with enzyme monomers begins at approximately 10 times lower calcium concentration than is required for calmodulin's stimulation of Ca^{2+}-ATPase activity. Half-maximal binding of calmodulin occurs at 0.09 μM Ca^{2+}, whereas half-maximal enzyme activation occurs at 0.25 μM Ca^{2+}. The calcium dependence of calmodulin binding to monomeric Ca^{2+}-ATPase is not cooperative (n_H = 1.1), while the calcium dependence of monomer activation does show positive cooperativity (n_H = 2.7) (Table I). As shown in Table I, the Ca^{2+} dependence of monomeric Ca^{2+}-ATPase activity in the absence of calmodulin is characterized by more than

2 times lower affinity for calcium and lack of cooperativity relative to the presence of calmodulin [see also Kosk-Kosicka and Inesi (1985)]. These findings are consistent with an order of events such that enzyme monomers are active at calcium concentrations below 10^{-6} M only after calmodulin binding. The Ca^{2+}, calmodulin-dependent stimulation of Ca^{2+}-ATPase activity is significant only after calmodulin binding has reached its half-maximal value, above 0.25 μM Ca^{2+}.

Contrary to the monomeric form, the oligomers show an almost identical calcium dependence of CaM binding and Ca^{2+}-ATPase activity (Figure 3B and Table I). Both reactions are half-maximal at 0.04–0.05 μM calcium and highly cooperative, with a n_H = 2.3–2.9. In agreement with previous reports, oligomers are activated at lower calcium concentrations than monomers, and their Ca^{2+}-ATPase activity is the same in the presence and absence of calmodulin [Table I and Kosk-Kosicka and Inesi (1985)]. Interestingly, the patterns of Ca^{2+} dependence of calmodulin binding and Ca^{2+}-ATPase activation (Figure 3B) are identical with Ca^{2+} binding curves obtained by titration of the tryptophan fluorescence intensity of the oligomeric enzyme (Kosk-Kosicka & Inesi, 1985). These findings suggest that the oligomerized Ca^{2+}-ATPase is in a conformation necessary for efficient, cooperative calcium binding at nanomolar Ca^{2+}, which the monomeric enzyme can acquire only upon interaction with calmodulin (Figure 3A).

Addition of the substrate, 3 mM ATP, with equivalent magnesium does not affect calcium dependence of calmodulin binding to either monomers or oligomers (Figure 3A,B).

Stoichiometry of Calmodulin Binding to the Ca^{2+}-ATPase. The monomeric and oligomeric enzyme was titrated with MIANS–CaM as shown in Figure 4. Aliquots of calmodulin were added to the 12 or 60 nM Ca^{2+}-ATPase where the equilibrium is shifted either toward monomers or toward oligomers, respectively. Comparison of the experimental data points shows significant differences in calmodulin binding to the two enzyme forms; the stoichiometry of calmodulin/Ca^{2+}-ATPase is 2 times lower for the oligomeric enzyme (Figure 4A). The data points were fitted by using an estimated K_d of 2.5 nM, in agreement with 1.4 times lower affinity for monomer activation by MIANS–CaM compared to bovine CaM which has a K_d of 1.6 nM [see Figure 1 and references in Kosk-Kosicka and Bzdega (1988)]. For the predominantly monomeric enzyme, the data were best fit by a theoretical curve assuming a 1:1 stoichiometry of calmodulin for Ca^{2+}-ATPase whereas for the predominantly oligomeric enzyme the best fit was obtained by assuming a 0.5:1 stoichiometry of calmodulin for Ca^{2+}-ATPase (Figure 4A). In the several titrations performed with seven different oligomeric Ca^{2+}-ATPase preparations, these values were very reproducible, with the stoichiometry ranging between 0.48:1 and 0.58:1 for calmodulin binding to the oligomers. The same stoichiometry was found for 45 and 75 nM enzyme, i.e., at two other concentrations which result in maximal Ca^{2+}-ATPase oligomerization (Kosk-Kosicka & Bzdega, 1988).

Discussion

MIANS–CaM was used to study the relationship between calmodulin binding and calmodulin activation of purified erythrocyte Ca^{2+}-ATPase. Our studies indicate that both native spinach calmodulin and MIANS–CaM stimulate monomeric Ca^{2+}-ATPase activity, although they are slightly less effective (estimated K_d = 2.5 nM) than bovine calmodulin. These slight differences in enzyme activation may be yet another example of reported differences in the interaction between phylogenetically different calmodulins and various target enzymes (Putkey et al., 1986; Roberts et al., 1984).

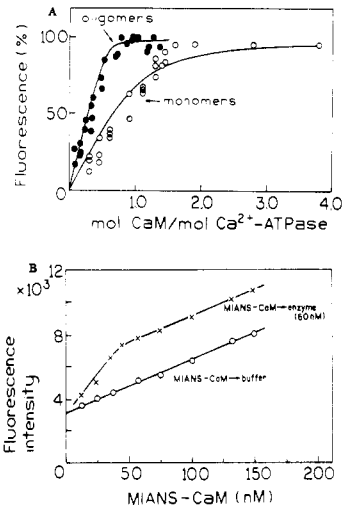

FIGURE 4: Calmodulin binding to enzyme monomers and oligomers. The fluorescence increase that occurs when predominantly monomeric (○) or oligomeric (●) enzyme was titrated with MIANS–CaM is shown as a function of the CaM:Ca^{2+}-ATPase molar ratio. MIANS–CaM titrations of the Ca^{2+}-ATPase, at either 12 (○) or 60 nM (●) concentration, and of the buffer alone were conducted. 100% fluorescence change is equivalent to a 47% and 53% increase of MIANS–CaM fluorescence intensity upon its reaction with enzyme monomers and oligomers, respectively, at saturating MIANS–CaM concentrations. Experimental conditions were identical with those in Figure 3, with no ATP added. Free calcium concentration was 17.75 μM. The data were fitted, assuming three different stoichiometries of calmodulin binding to the two enzyme forms. For clarity, only the best fits are shown, obtained by assuming a 0.5:1 and a 1:1 stoichiometry of CaM binding to the predominantly oligomeric and monomeric Ca^{2+}-ATPase, respectively. Equation 1, described under Materials and Methods, was used. Panel B shows an example of calmodulin titrations of Ca^{2+}-ATPase compared to the control calmodulin titrations of the buffer. The latter produces a linear increase in fluorescence as would be expected as the concentration of the fluorescent calmodulin is increased. The data points presented in panel A were derived from five sets of similar data where the calmodulin titration in buffer was subtracted from the calmodulin titration of the Ca^{2+}-ATPase.

The Ca^{2+}-ATPase exists in equilibria between calmodulin-dependent monomers and fully active oligomers. Half-maximal oligomerization occurs at 15–20 nM Ca^{2+}-ATPase (Kosk-Kosicka & Bzdega, 1988). Previous findings showed that both monomeric and oligomeric enzyme forms bind calmodulin (Kosk-Kosicka & Bzdega, 1988). While calmodulin binding to monomers resulted in enzyme activation, calmodulin binding to oligomers produced no effect on Ca^{2+}-ATPase activity. Calmodulin's failure to alter the activity of oligomeric enzyme could result from alterations in calmodulin's affinity, stoichiometry, ligand dependence, or binding site on the oligomerized enzyme. Indeed, our present studies demonstrate that both the Ca^{2+} dependence and stoichiometry of CaM's interaction with enzyme monomers are different from its interaction with enzyme oligomers (Figures 3 and 4).

Differences in Ca^{2+} Dependence of Calmodulin Binding to Oligomeric and Monomeric Enzyme. MIANS–CaM binds to the oligomer at a 2 times lower concentration of Ca^{2+} and with greater cooperativity, as a function of [Ca^{2+}] (n_H = 2.3 compared to n_H = 1.1), than to the monomer. The differences observed in the calcium dependence of MIANS–CaM binding to the monomers and oligomers of Ca^{2+}-ATPase suggest that calmodulin interacts differently and perhaps exhibits a different conformation (and Ca^{2+} affinity) when bound to monomeric than when bound to oligomeric enzyme. Consistent with this, Cox et al. (1982) have shown that the conformation of calmodulin and the cooperativity of its Ca^{2+} binding are

strongly influenced by the nature of its interface with target proteins. Calmodulin binding to monomers would be expected to induce conformational changes in calmodulin which alter its Ca^{2+} affinity, and conformational changes in the Ca^{2+}-ATPase which result in its activation. Calmodulin binding to oligomers would be expected to produce slightly different conformational changes in calmodulin (greater increase in cooperativity of Ca^{2+} binding) but less significant changes in Ca^{2+}-ATPase conformation since it is already in an active state by virtue of oligomerization. Consistent with this, calmodulin dramatically increases fluorescein 5′-isothiocyanate mobility in Ca^{2+}-ATPase monomers but has little effect on probe mobility in oligomers (Kosk-Kosicka & Bzdega, 1988). At this point, it is not clear if the conformation of the oligomers is same as that of the calmodulin-activated monomers, although this is expected from the similarity of the activity of the two forms.

Differences in Stoichiometry of Calmodulin Binding to Oligomeric and Monomeric Enzyme. There is a 2-fold difference in the stoichiometry of calmodulin binding to the two enzyme forms (Figure 4A). Interaction of MIANS–CaM with oligomers saturates near 0.5 mol of calmodulin per 1 mol of enzyme. The data obtained from several fluorescence titrations of various enzyme preparations are very consistent in establishing this stoichiometry. Such a stoichiometry suggests binding of 1 mol of calmodulin per two associated enzyme molecules. If the associated enzyme is dimeric, then one calmodulin binding site on the monomer must be lost upon enzyme–enzyme interaction, possibly by becoming part of the interface of the oligomerized molecules. The loss of one calmodulin binding site per two Ca^{2+}-ATPase molecules and a "calmodulin-like" activation of the oligomeric enzyme suggest that a calmodulin binding site might be involved in the oligomerization process. Consistent with this hypothesis is the finding that calmodulin apparently decreases the enzyme's ability to oligomerize (Kosk-Kosicka & Bzdega, 1990). Thus far, no information is available on the regions of the Ca^{2+}-ATPase molecule involved in the oligomerization reaction. Recently, the primary sequence of one CaM binding site, located in the C-terminal of this enzyme, has been reported (James et al., 1988; Verma et al., 1988). It would be of great interest to determine if this region is involved in the oligomerization reaction.

Our MIANS–CaM titrations of monomeric enzyme (12 nM) are best fit by assuming a stoichiometry of CaM/Ca^{2+}-ATPase of 1:1. This is consistent with our previous finding that monomers (15 nM) are fully activated upon the binding of 1 mol of CaM/1 mol of Ca^{2+}-ATPase (Kosk-Kosicka & Bzdega, 1988). Other authors' studies (analysis of activation, cross-linking with azidocalmodulin, or binding of iodinated calmodulin) pointed to either one (Graf & Penniston, 1981) or two [see James et al. (1988)] CaM binding sites per enzyme molecule. Our present study resolves this discrepancy by following directly the binding of MIANS–CaM to the Ca^{2+}-ATPase and by taking into account the affinity of calmodulin for the enzyme, as well as the oligomeric state of the Ca^{2+}-ATPase.

In conclusion, our data demonstrate significant differences in the Ca^{2+} dependence and stoichiometry of calmodulin binding to the oligomeric and to the monomeric enzyme. It appears that one of the CaM binding sites on the Ca^{2+}-ATPase may be buried upon enzyme oligomerization, resulting in conformational changes and full catalytic activation of the Ca^{2+}-ATPase. Our studies relating the calcium dependence of calmodulin binding to the calcium dependence of calmodulin activation of the monomeric enzyme are consistent with the thought that calmodulin is a biochemical activator of the Ca^{2+}-ATPase within the cell. In this sense, a rise in cytosolic calcium concentration would result in calmodulin binding to monomeric enzyme to produce its activation and to enhance calcium extrusion from the cell. Whether or not enzyme oligomerization occurs in normal or pathological states in cells has yet to be determined, but if it does, it could certainly produce alterations in cellular calcium homeostasis.

Registry No. ATPase, 9000-83-3; Ca, 7440-70-2.

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