Effects of Calmodulin on Erythrocyte Ca^{2+}-ATPase Activation and Oligomerization†

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ABSTRACT: The study was performed on the purified human erythrocyte Ca^{2+}-ATPase to test whether or not calmodulin promotes enzyme oligomerization. Two physiologically significant modes of activation of this enzyme were considered, by calmodulin binding to monomeric enzyme and by enzyme oligomerization [Kosk-Kosicka & Bzdega (1988) *J. Biol. Chem. 263*, 18184]; it was not clear whether the two modes were interdependent or operated independently. Fluorescence resonance energy transfer (FRET) between separately labeled Ca^{2+}-ATPase molecules was used to monitor oligomerization. No change in energy transfer efficiency was observed upon subsequent addition of calmodulin at different enzyme concentrations. Lack of decrease in the enzyme concentration at which the half-maximal oligomerization occurred indicated that calmodulin did not facilitate oligomerization. The calmodulin inhibitor compound 48/80 had no effect on either the Ca^{2+}-ATPase activity of oligomers or the extent of oligomerization measured by FRET while it drastically decreased the calmodulin-stimulated activity of the monomeric Ca^{2+}-ATPase. The findings demonstrate that calmodulin is not involved in the oligomerization-induced activation pathway; it neither promotes oligomerization nor stimulates the Ca^{2+}-ATPase activity of oligomers. We have demonstrated that calmodulin added before mixing donor- and acceptor-labeled enzyme populations prevented the occurrence of energy transfer. This inhibition of the formation of mixed donor–acceptor enzyme oligomers by calmodulin was dose dependent. Also, the reversal of the inhibition by compound 48/80 proceeded in a dose-dependent manner. Further, calmodulin prevented the apparent decrease of energy transfer efficiency that resulted from dilution of mixed donor–acceptor enzyme oligomers with unlabeled enzyme. These observations suggest that binding of calmodulin to both monomeric and oligomeric Ca^{2+}-ATPases temporarily locks the enzyme in its conformation. It appears then that the equilibrium between enzyme monomers and oligomers, and the availability of calmodulin to these two enzyme forms, determines the activation pathway of the Ca^{2+}-ATPase.

The erythrocyte Ca^{2+}-ATPase was the first plasma membrane enzyme shown to work as a Ca^{2+} pump (Schatzmann, 1966). Later it was demonstrated that the enzyme binds calmodulin and is activated by it (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977). As the concentration of calmodulin in the red cell is well in excess over that of the Ca^{2+}-ATPase, it is not clear exactly how the activation by calmodulin proceeds, other than that it is Ca^{2+} concentration dependent. Recently we have proposed yet another pathway of Ca^{2+}-ATPase activation, by self-association of the enzyme to form oligomers (Kosk-Kosicka & Bzdega, 1988).

At present, we are considering two models to explain the observed modes of activation of the erythrocyte Ca^{2+}-ATPase taking into account the following observations: (1) the detergent-solubilized Ca^{2+}-ATPase exists in equilibria between monomeric and oligomeric forms (Kosk-Kosicka & Bzdega, 1988); (2) both modes of Ca^{2+}-ATPase activation, by interaction of monomers with calmodulin and by enzyme oligomerization, occur at physiologically relevant Ca^{2+} concentrations (Kosk-Kosicka et al., 1989, 1990b); (3) the oligomerization process and the resulting activation can occur in the absence of calmodulin (Kosk-Kosicka & Bzdega, 1988; Kosk-Kosicka et al., 1989); (4) calmodulin binds to both enzyme forms; however, the Ca^{2+} dependence and stoichiometry of the binding are different (Kosk-Kosicka & Bzdega, 1988; Kosk-Kosicka et al., 1990b). Our first model assumes two active enzyme species: monomers with bound calmodulin and oligomers. They are equally active as revealed by Ca^{2+}-ATPase activity. According to the second model, only oligomers are fully active. Calmodulin increases the affinity between enzyme molecules and facilitates oligomerization [for a discussion, see also Kosk-Kosicka and Bzdega (1988)]. As such, calmodulin does not activate directly, but only as a secondary effect in promoting enzyme oligomerization.

In the present study, we sought to clarify whether or not calmodulin is involved in the mode of activation by oligomerization. In particular, we asked if calmodulin facilitates oligomerization, and if the two activation modes are interdependent. We took advantage of the fluorescence energy transfer (FRET)[1] technique that was established for studies of the Ca^{2+}-ATPase oligomerization (Kosk-Kosicka et al., 1989), as well as of the availability of an anti-calmodulin drug which specifically inhibited calmodulin. Both methods have proven very valuable in differentiating between the calmodulin-dependent and calmodulin-independent activation modes.

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[1] Abbreviations: $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; FITC, fluorescein 5′-isothiocyanate; TRITC, tetramethylrhodamine 5-isothiocyanate; FRET, fluorescence resonance energy transfer.

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The generated results are in agreement with our first model and demonstrate that calmodulin's action does not involve a change in the degree of association between Ca^{2+}-ATPase molecules.

Materials and Methods

Egg yolk phosphatidylcholine (P5763) and CNBr-activated Sepharose 4B were purchased from Sigma; $C_{12}E_8$ was from Nikkol, Japan. Coupling of bovine calmodulin to Sepharose was performed in accordance with Pharmacia instructions, as described earlier (Kosk-Kosicka et al., 1986).

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 7.5, 130 mM KCl, 0.5 mM $MgCl_2$, 5 mM EGTA, 20% glycerol, 750 μM $C_{12}E_8$, 2 mM dithiothreitol, 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 method of Lowry et al. (1951) as modified by 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 gave similar values within 5% error. Bovine serum albumin was used as a standard. The Ca^{2+}-ATPase molecular weight of 140000 determined by gel electrophoresis was used to calculate mole amounts of the protein (Kosk-Kosicka et al., 1986).

Ca^{2+}-ATPase Activity. Ca^{2+}-ATPase activity was determined by measurement of inorganic phosphate production, as described previously (Kosk-Kosicka & Bzdega, 1988). The assay was performed in a reaction mixture containing 50 mM Tris–maleate, pH 7.4, 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 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 reaction was started with 3 mM ATP and carried out for up to 30 min at 37 °C. Aliquots were withdrawn at various times for colorimetric inorganic phosphate measurement with either malachite green (Lanzetta et al., 1979) or ammonium molybdate/metavanadate (Lin & Morales, 1977). Steady-state velocities were obtained from plots of inorganic phosphate production which were linear with time.

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

Fluorescence Labeling. The Ca^{2+}-ATPase in erythrocyte ghost preparation was labeled with fluorescein 5′-isothiocyanate (FITC) or tetramethylrhodamine 5-isothiocyanate (TRITC) in the dark at 20 °C in a medium containing 130 mM KCl, 10% glycerol, the protease inhibitor aprotinin (100 kallikrein units/mL), and 10 mM Tris-HCl, pH 9.0. Ghost protein (3.5 mg/mL) was incubated for 20 min with 15 μM FITC; 1.75 mg/mL ghost protein was incubated for 30 min with 100 μM TRITC. The unreacted probe was removed by two washings in 5 volumes of a medium containing 10 mM Tris–maleate, pH 7.5, 130 mM KCl, 0.5 mM $MgCl_2$, 50 μM $CaCl_2$, and 10% glycerol. The labeled Ca^{2+}-ATPase was then purified by our standard procedure described above. The stoichiometry of labeling was measured after the purified enzyme was eluted from the Cam–Sepharose column [for details on enzyme purification, see Kosk-Kosicka and Inesi (1985) and Kosk-Kosicka et al. (1986)]. $\epsilon_{552} = 9.6 \times 10^4\ M^{-1}\ cm^{-1}$ was used for the TRITC-labeled enzyme and $\epsilon_{495} = 8 \times 10^4\ M^{-1}\ cm^{-1}$ for the FITC-labeled enzyme, in the presence of 0.1% NaOH (Mitchinson et al., 1982).

Fluorescence Measurements. Fluorescence excitation and emission spectra of the FITC–Ca^{2+}-ATPase and TRITC–Ca^{2+}-ATPase were recorded with an SLM-8000 spectrofluorometer, using excitation and emission slits of 8 nm. Excitation and emission wavelengths used for the two fluorescently labeled Ca^{2+}-ATPase preparations were 470 and 525, 550 and 570 nm, respectively. Aliquots of the labeled enzyme, from 5 to 100 μL, were added to 0.9 mL of reaction mixture and supplemented with required aliquots of elution buffer so that the total volume was always =1 mL. The reaction mixture composition was the same as in Ca^{2+}-ATPase assays, except ATP was omitted. The concentration of $C_{12}E_8$ was kept constant at 150 μM.

Compound 48/80 was dissolved in ethanol. Small aliquots of the stock solution of 10 mg/mL were added to the reaction mixture such that final concentrations of ethanol did not exceed 0.5% in fluorescence measurements and 3% in Ca^{2+}-ATPase activity assays. Ethanol at these concentrations did not affect Ca^{2+}-ATPase activity.

All measurements were done in triplicate, and the presented data points are averages of three to five experiments. The individual measurements did not differ from the mean more than 15%.

Results

Effect of Calmodulin on Energy Transfer Efficiency

Enzyme Concentration Dependence of FRET. Previously we have established FRET as a direct physical method of measuring Ca^{2+}-ATPase oligomerization (Kosk-Kosicka et al., 1989). Energy transfer efficiency was calculated from the decrease in fluorescence intensity of the FITC (donor-labeled enzyme) at 525 nm observed upon addition of the acceptor-labeled enzyme (eosinyl-5-maleimide or TRITC); half-maximal oligomerization was determined at 10–12 nM enzyme (Kosk-Kosicka et al., 1989). To test whether or not calmodulin affected Ca^{2+}-ATPase oligomerization, the energy transfer efficiency between the FITC-labeled and TRITC-labeled Ca^{2+}-ATPase molecules was measured at different total enzyme concentrations (Figure 1). In the absence of calmodulin, half-maximal oligomerization was observed at 10 nM Ca^{2+}-ATPase, similar to the previous reports. Subsequent addition of calmodulin at any given enzyme concentration had no effect on the energy transfer efficiency, neither on the half-maximal nor on the maximal level of the dependence shown in its absence (Figure 1).

The fact that calmodulin did not increase energy transfer efficiency at low enzyme concentrations indicates that calmodulin does not facilitate enzyme oligomerization. At the same time, lack of a visible effect of calmodulin at higher enzyme concentrations shows that calmodulin does not dissociate oligomers to a significant extent.

Calmodulin Concentration Dependence of FRET. To test whether or not the calmodulin concentration was limiting, the energy transfer efficiency was measured at different calmodulin concentrations using 24 nM total Ca^{2+}-ATPase. At this concentration, the enzyme was predominantly oligomeric as evidenced by maximal energy transfer efficiency in Figure 1. Figure 2 shows that addition of up to 480 nM calmodulin to

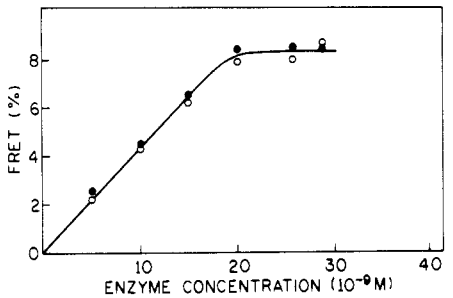

FIGURE 1: Enzyme concentration dependence of energy transfer efficiency in the presence (●) and absence (○) of calmodulin. The enzyme was labeled with either FITC or TRITC in red blood cell ghosts as described under Materials and Methods and subsequently purified in the standard procedure (Kosk-Kosicka & Inesi, 1985; Kosk-Kosicka et al., 1986). The efficiency of FRET was calculated from the decrease of FITC emission of the FITC-labeled enzyme (donor) at 525 nm occurring upon addition of an equal amount of TRITC-labeled enzyme (acceptor) (Kosk-Kosicka et al., 1989). The measurements were performed at different total (FITC-labeled plus TRITC-labeled) enzyme concentrations indicated on the x axis. The decrease of FITC emission was expressed as percent of the fluorescence intensity observed before addition of acceptor-labeled enzyme. Controls showed that addition of an equal amount of unlabeled enzyme did not cause any decrease of FITC emission of the FITC-labeled enzyme. Dilution effect equal to 1% has been subtracted. The assay medium is described under Materials and Methods. Free [Ca^{2+}] was 17 μM. Calmodulin when present was 100 nM and was added after both donor- and acceptor-labeled populations of enzyme were present in the assay.

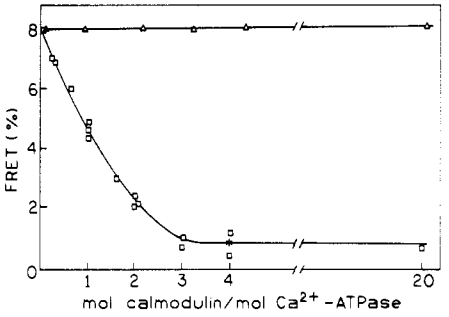

FIGURE 2: Calmodulin concentration dependence of energy transfer. The assay was performed as described in Figure 1. The energy transfer occurring between the FITC- and the TRITC-labeled enzyme molecules was measured. The *total* enzyme protein concentration was constant at 24 nM (□, △), except for the 56 nM total enzyme in the experiment when calmodulin was added to 28 nM FITC–enzyme (asterisks). Calmodulin was added either at high (24 nM total) enzyme concentration, when both the FITC-labeled and TRITC-labeled enzyme populations were present (△) or when only 28 nM FITC-labeled enzyme was present (asterisks), or at low enzyme concentration, when only FITC-labeled, 12 nM, enzyme was present (□). The FITC-enzyme was preincubated with calmodulin for 3 min before addition of TRITC–enzyme. Calmodulin concentrations were appropriate to yield calmodulin to total enzyme molar ratios indicated on the x axis.

the mixed FITC-labeled plus TRITC-labeled enzyme had no effect on the observed energy transfer efficiency. However, an apparent reduction in energy transfer efficiency was observed if calmodulin was added to the 12 nM FITC-labeled, significantly monomeric enzyme before an equal amount of TRITC-labeled enzyme. In such a case, up to 90% of energy transfer could be eliminated by 75 nM calmodulin (calmodulin to total enzyme ratio of 3:1). Further increase in calmodulin concentration did not result in any further apparent decrease of energy transfer. Half-maximal inhibition of oligomerization between FITC-labeled and TRITC-labeled enzyme molecules occurred at a 1:1 calmodulin to total enzyme ratio.

Specificity of Calmodulin Effect. The dose dependency of calmodulin's effect in Figure 2 conforms to its specificity. Additionally, bovine serum albumin substituted for calmodulin

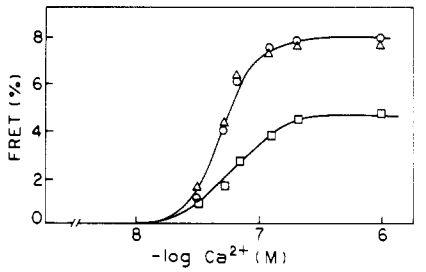

FIGURE 3: Ca^{2+} concentration dependence of energy transfer in the absence (○) and presence of calmodulin added to 12 nM FITC-labeled enzyme either before (□) or after addition of 12 nM TRITC–enzyme (△). Total Ca^{2+}-ATPase concentration was 24 nM; calmodulin concentration was 30 nM. Various amounts of Ca^{2+} were added to obtain the free Ca^{2+} concentration indicated on the horizontal axis. EGTA concentration was 1 mM. FRET was measured as in Figure 1.

had no effect on the observed energy transfer efficiency, independent of whether it was added to the FITC-labeled enzyme before or after the TRITC-labeled enzyme.

Next we tested whether calmodulin caused the apparent decrease in energy transfer efficiency because it was added to significantly monomeric enzyme or because only FITC-labeled enzyme (i.e., homogeneous enzyme population with respect to the label attached) was present at the time of its addition (Figure 2). To differentiate between these two possibilities, calmodulin was added to the oligomeric, FITC-labeled enzyme at the highest possible (28 nM) enzyme concentration, and following addition of an equal amount of the TRITC-labeled enzyme, the energy transfer was studied. As shown in Figure 2, the energy transfer efficiency was comparable to that observed when calmodulin was added to the significantly monomeric FITC-labeled enzyme. In yet another approach, mixed FITC/TRITC 24 nM oligomeric enzyme was diluted by addition of unlabeled 10 nM Ca^{2+}-ATPase. This caused a 30% decrease of energy transfer efficiency. However, there was no decrease in energy transfer efficiency if the unlabeled enzyme was added after calmodulin (not shown). Altogether these observations suggest that calmodulin preserved the existing monomer–oligomer equilibrium such that formation of mixed donor–acceptor-labeled or labeled–unlabeled enzyme oligomers was prevented or delayed.

Ca^{2+} Concentration Dependence of FRET. We have previously demonstrated in FRET studies of the FITC–enzyme and eosinyl-5-maleimide-labeled enzyme pair that oligomerization of the Ca^{2+}-ATPase was Ca^{2+} dependent and closely resembled the Ca^{2+} concentration dependence of the Ca^{2+}-ATPase activity, with a K_{Ca} in the range of 7.15–7.45 pCa (Kosk-Kosicka & Inesi, 1985; Kosk-Kosicka et al., 1989, 1990b). To determine whether or not calmodulin affected the observed Ca^{2+} concentration dependence, we proceeded with studies of the Ca^{2+} concentration dependence of energy transfer efficiency from FITC- to TRITC-labeled enzyme molecules in the presence and absence of calmodulin. For this purpose, calmodulin was used at a concentration at which it prevented oligomerization by 50% so the effect of calmodulin on Ca^{2+} affinity could be monitored. As shown in Figure 3, the [Ca^{2+}] dependence in the absence of calmodulin resembled very closely that of the FITC/eosinyl-5-maleimide pair (Kosk-Kosicka et al., 1989) with K_{Ca} = 7.3, confirming that FRET is revealing the [Ca^{2+}] dependence of oligomerization, being independent of the label attached to the enzyme. The presence of calmodulin caused a consistent decrease in the transfer efficiency at all Ca^{2+} concentrations studied (about 50%). No significant change in Ca^{2+} affinity by calmodulin was observed.

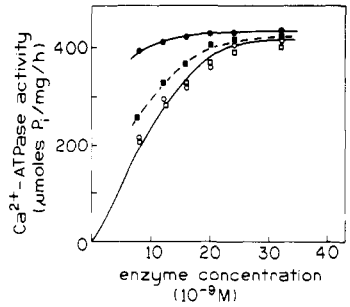

FIGURE 4: Enzyme concentration dependence of the inhibition of Ca^{2+}-ATPase activity by compound 48/80. When present, compound 48/80 was at a concentration of 50 μg/mL (□, ■). The Ca^{2+}-ATPase activity assay was performed as described under Materials and Methods in either the absence (open symbols) or the presence (solid symbols) of 100 nM calmodulin. Free [Ca^{2+}] was 17 μM. The calmodulin-dependent Ca^{2+}-ATPase activity and the calmodulin-independent Ca^{2+}-ATPase activity represent enzyme monomers and oligomers, respectively.

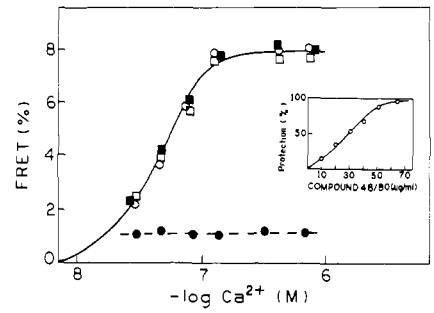

FIGURE 5: Prevention of calmodulin effect on energy transfer by compound 48/80. The assay was performed in either the absence (open symbols) or the presence (solid symbols) of 100 nM calmodulin added to 12 nM FITC–enzyme. Total enzyme concentration was 24 nM (12 nM FITC- plus 12 nM TRITC-labeled enzyme). The concentration of compound 48/80 was 50 μg/mL (□, ■). Free [Ca^{2+}] was 17 μM. The inset shows how compound 48/80 blocks calmodulin from interacting with the enzyme. Protection of energy transfer efficiency is expressed in percent as a function of the concentration of compound 48/80.

Effect of Compound 48/80 on Ca^{2+}-ATPase Activity of Monomers and Oligomers

The results showing that calmodulin does not facilitate enzyme oligomerization (Figures 1–3) are in line with our previous findings that calmodulin does not stimulate the Ca^{2+}-ATPase activity of oligomers (Kosk-Kosicka & Bzdega, 1988). These data strongly suggest that no step in the activation by oligomerization pathway is stimulated by calmodulin. We have employed calmodulin inhibitors in both Ca^{2+}-ATPase activity and FRET studies to further examine this issue.

Dependence on Calmodulin Inhibitor Concentration. Studies of the effect of different anti-calmodulin drugs on the Ca^{2+}-ATPase activity have shown that compound 48/80 is the most specific inhibitor of the calmodulin-dependent Ca^{2+}-ATPase activity of the monomeric enzyme (unpublished data; Kosk-Kosicka et al., 1990a). The half-maximal inhibition occurred at 2 μg of 48/80 per 1 mL, with the maximal effect at 50 μg/mL. In contrast, compound 48/80 did not inhibit the Ca^{2+}-ATPase activity of oligomers, which is independent of calmodulin (see also Figure 4). Thus, compound 48/80 inhibits only the calmodulin-dependent Ca^{2+}-ATPase activity, showing that indeed it is a good inhibitor of calmodulin and that it has no unspecific effect on the Ca^{2+}-ATPase activity itself. It was selected then as a very useful tool to differentiate between calmodulin-dependent and calmodulin-independent processes in Ca^{2+}-ATPase activation.

Dependence on Enzyme Concentration. The effect of compound 48/80 on the two modes of Ca^{2+}-ATPase activation is compared in Figure 4. The Ca^{2+}-ATPase activity was assayed as a function of enzyme concentration either in the presence (activation of Ca^{2+}-ATPase monomers by calmodulin) or in the absence of calmodulin (enzyme activation by oligomerization), with or without addition of compound 48/80 at the concentration at which it maximally inhibited calmodulin stimulation. The results in Figure 4 show that in the presence of compound 48/80 activation by calmodulin was reduced at all enzyme concentrations studied, and approached the level of Ca^{2+}-ATPase activity characteristic of the oligomeric enzyme. Contrary to the activation of monomers by calmodulin, the Ca^{2+}-ATPase activity of oligomers was not affected at any enzyme concentration.

Effect of Compound 48/80 on Energy Transfer

Subsequently the effect of compound 48/80 on the level of enzyme oligomerization was studied by FRET to evaluate the hypothesis according to which the decrease observed in Figure 4 in calmodulin-stimulated activation resulted from calmodulin becoming unavailable to the Ca^{2+}-ATPase upon binding of compound 48/80. This removal of calmodulin was in turn expected to increase the extent of oligomerization. Figure 5 shows that in the presence of compound 48/80 the energy transfer (measured for 12 nM donor- plus 12 nM acceptor-labeled enzyme) was always equal to that measured without any calmodulin added. Thus, indeed calmodulin inhibitor totally abolished the inhibiting effect of calmodulin on enzyme oligomerization (Figures 2 and 3). The concentration dependence of the effect of 48/80 on the extent of oligomerization, as measured by FRET (Figure 5, inset), was quite similar to its effect on the Ca^{2+}-ATPase activity showing that the two processes studied with compound 48/80, oligomerization and oligomerization-induced Ca^{2+}-ATPase activity, are closely related features of the enzyme.

Discussion

Calmodulin Does Not Stimulate Oligomerization-Induced Activation of the Ca^{2+}-ATPase. We considered two models to explain the role of calmodulin in Ca^{2+}-ATPase activation: direct activation of enzyme monomers upon binding of calmodulin or an indirect effect of calmodulin, by facilitating enzyme oligomerization. Previous studies could not discriminate between these two models. A role for calmodulin in promoting oligomerization was suggested by a 1.8 times lowered enzyme concentration required for half-maximal Ca^{2+}-ATPase activity in its presence (Kosk-Kosicka & Bzdega, 1988). By monitoring Ca^{2+}-ATPase activity alone, we could not test this postulated role for calmodulin as its activation of monomers resulted in a Ca^{2+}-ATPase activity very similar to the activation by enzyme self-association. Additionally, some other evidence pointed to lack of involvement of calmodulin in Ca^{2+}-ATPase activation of oligomers. For example, calmodulin added to oligomers did not cause significant stimulation of Ca^{2+}-ATPase activity; there were no traces of contaminating calmodulin in the enzyme preparation, and the Ca^{2+} concentration dependence of the two activation pathways differed significantly (Kosk-Kosicka et al., 1986, 1990b). Fluorescence polarization studies of the FITC-labeled enzyme were employed which did not confirm calmodulin's role in facilitation of oligomerization. However, the experiment was not considered conclusive because of the short lifetime limitations of the fluorescent probe used [discussed in Kosk-Kosicka and Bzdega (1988)]. To clarify this issue in the present study, we have utilized a FRET technique whose usefulness as a direct physical method of measuring the oligomerization of the erythrocyte Ca^{2+}-ATPase and determining factors that

affect this process was recently established (Kosk-Kosicka et al., 1989).

We have shown that addition of calmodulin to mixed donor/acceptor-labeled enzyme populations did not affect energy transfer efficiency at any enzyme concentration studied (Figure 1). A shift was expected in the half-maximal enzyme concentration toward lower values if calmodulin facilitated the oligomerization by increasing the affinity between enzyme molecules. The fact that such a shift did not occur (Figure 1) establishes that calmodulin has no role in facilitating oligomerization. This finding was confirmed in the following studies using compound 48/80 (Figures 4 and 5). Under our conditions, 48/80 proved to be a specific inhibitor of calmodulin that did not affect the Ca^{2+}-ATPase, which is in agreement with the reports on studies of the Ca^{2+}-ATPase activity of the erythrocyte Ca^{2+}-ATPase in membranous preparations (Gietzen et al., 1981; Rossi et al., 1985). Compound 48/80 had a dramatic effect on the Ca^{2+}-ATPase activity of monomers, reducing it by over 90% in a dose-dependent manner, while it had no effect on the Ca^{2+}-ATPase activity in the absence of calmodulin (Figure 4). There was a very good agreement between Ca^{2+}-ATPase activity (Figure 4) and FRET (Figure 5) data; compound 48/80 did not affect either the extent of enzyme oligomerization or the level of Ca^{2+}-ATPase activity of oligomers. The present data confirm several previous indications that calmodulin is not involved in the activation by oligomerization and indicate that calmodulin does not act as a promoter of enzyme oligomerization. Actually, oligomerization-induced activation of the enzyme is not stimulated by calmodulin at any step studied (Figures 1–4). Thus, calmodulin activates the enzyme only directly upon binding to monomers.

Interdependence of the Two Ca^{2+}-ATPase Activation Pathways. The dose-dependent manner of the inhibition of enzyme oligomerization by calmodulin (Figures 2 and 3) and of the reversal of this inhibition by compound 48/80 (Figure 5), as demonstrated in FRET studies, show a direct correlation between the availability of calmodulin to the enzyme and the inhibition of enzyme oligomerization. At first, it seemed that calmodulin by binding to monomers shifted the monomer–oligomer equilibrium toward monomers, thus causing the observed reduction in the level of oligomerization (Figure 2). However, this explanation does not agree with the absence of the decrease in energy transfer efficiency when calmodulin is added to the significantly monomeric mixed donor/acceptor-labeled enzyme population (Figure 1). More probably, calmodulin by binding to both monomeric and oligomeric forms of the enzyme locks them in their states for a time which is on the order of the duration of the experiment or longer, thus stabilizing the enzyme conformation. There is some strong even though indirect evidence to back this explanation. First of all, calmodulin added to the FITC-labeled oligomeric enzyme prevents energy transfer like it does when added to the FITC-labeled 50% monomeric enzyme (Figure 2). Thus, it is not a calmodulin–(FITC)monomer species that prevents formation of mixed FITC/TRITC oligomers but rather calmodulin–enzyme (calmodulin–monomers and calmodulin–oligomers) that prevents or delays associations between the FITC-labeled and the TRITC-labeled enzyme molecules. Further, addition of unlabeled enzyme to the mixed FITC-labeled/TRITC-labeled enzyme apparently decreases energy transfer efficiency as expected upon dilution of the FITC/TRITC–enzyme population. However, if calmodulin is added prior to the unlabeled enzyme, the dilution of the FITC/TRITC enzyme does not cause a decrease in energy transfer efficiency (see Results).

Assuming that calmodulin binds with the same affinity to both enzyme forms [K_d = 1.6 nM, as determined for monomers and bovine calmodulin in Kosk-Kosicka and Bzdega (1988)] and that its dissociation is significantly slower than its binding to the enzyme, and taking into account that only binding to monomers stimulates the Ca^{2+}-ATPase activity whereas binding to oligomers does not (Kosk-Kosicka & Inesi, 1985; Kosk-Kosicka & Bzdega, 1988; Kosk-Kosicka et al., 1990b), it appears that the extent of availability of calmodulin and also enzyme concentration in addition to Ca^{2+} concentration (Kosk-Kosicka et al., 1989) are determining factors in Ca^{2+}-ATPase activation. If calmodulin is available at high enough concentration (Figure 2), then at low enzyme concentration monomer–calmodulin species will prevail because the K_d for calmodulin–monomer association is 1 order of magnitude lower than the K_d for enzyme–enzyme associations (Kosk-Kosicka & Bzdega, 1988). As a result, at low enzyme concentrations, below K_d, calmodulin may drastically reduce activation by oligomerization through freezing. At high enzyme concentrations, when oligomerization is preferred, the presence of calmodulin does not exert any visible effect, as it probably freezes monomers and oligomers equally well. This freezing or locking mechanism could be well explained by conformational change caused in the enzyme molecule upon binding of calmodulin. As the monomer–calmodulin species does not oligomerize, it is highly probable that the calmodulin binding domain is required in enzyme–enzyme interactions.

In conclusion, calmodulin activates only monomers of the erythrocyte Ca^{2+}-ATPase. It does not stimulate activation by oligomerization. Unlimited availability of calmodulin to the enzyme at low concentration may drastically reduce activation by oligomerization.

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Molecular Cloning of cDNA for Proteasomes from Rat Liver: Primary Structure of Component C3 with a Possible Tyrosine Phosphorylation Site†,‡

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ABSTRACT: Proteasomes are multicatalytic proteinase complexes consisting of multiple components. Previously, we reported the cloning and sequencing of cDNA for the largest component, C2, of rat liver proteasomes [Fujiwara, T., Tanaka, K., Kumatori, A., Shin, S., Yoshimura, T., Ichihara, A., Tokunaga, F., Aruga, R., Iwanaga, S., Kakizuka, A., & Nakanishi, S. (1989) *Biochemistry 28*, 7332–7340]. In the present study, the nucleotide sequence of another component (C3) of proteasomes has been determined from a recombinant cDNA clone isolated by screening a rat liver cDNA library with synthetic oligodeoxynucleotide probes corresponding to partial amino acid sequences of the protein. The deduced sequence of component C3 consists of 234 amino acid residues with a calculated molecular weight of 25 925. These values are consistent with those obtained by protein chemical analyses. A single mRNA species hybridizing to the C3 cDNA of rat liver was expressed in all rat tissues examined and in a variety of other eukaryotic organisms, its distribution being similar to that of C2 mRNA. The wide distribution of the gene product, possibly C3, suggests that this protein functions similarly in most eukaryotes. C3 is an unmodified protein of a single gene and differs from any other known protein, but its overall amino acid sequence resembles those of other proteasomal components, including C2, suggesting that these components belong to a single family of proteins with the same evolutionary origin. Interestingly, a sequence of about 70 amino acid residues in the C3 protein is very similar to the amino acid sequences, including a conserved autophosphorylated tyrosine residue, present in various cellular tyrosine kinases such as *src* gene products and some receptor proteins; the amino acids in this sequence show about 30% identify with those of the sequences of tyrosine kinases. Conceivably, therefore, the C3 protein has a tyrosine phosphorylation site.

Proteasomes (multicatalytic proteinase complexes) are widely distributed in eukaryotes ranging from man to yeast (Tanaka et al., 1988a; Rivett, 1989). There is increasing evidence that they have ATP-dependent proteolytic activities (Waxman et al., 1985; Driscoll & Goldberg, 1989) and are involved in an ATP/ubiquitin-dependent nonlysosomal proteolytic pathway (Ganoth et al., 1988; McGuire et al., 1988; Tanaka & Ichihara, 1988; Matthews et al., 1989). Proteasomes were initially found in the cytoplasm of cells, but later they were demonstrated in the nucleus also at considerably high concentration (Arrigo et al., 1988; Tanaka et al., 1989). Interestingly, these proteasomes are similar in size, shape, and subunit structure to the 19S–22S ring-shaped particles that are ubiquitous in eukaryotes (Martins de Sa et al., 1986; Arrigo et al., 1987) and that are proposed to have critical cellular functions, such as in repressing mRNA translation (Martins de Sa et al., 1986) and tRNA processing (Castano et al., 1986). In fact, the identity of proteasomes as multicatalytic proteinases with these 20S particles was recently reported by us (Arrigo et al., 1988) and others (Falkenburg et al., 1988; Kleinschmidt et al., 1988). These 20S proteasomal particles appear to play an important role in development in the sea urchin (Akhayat et al., 1987), newt (Gounon et al., 1988), axolotl (Gautier et al., 1988), and *Drosophila* (Haass & Kloetzel, 1989; Haass et al., 1989), but their biological significance is still obscure.

Rat liver proteasomes are symmetrical ring-shaped particles with a sedimentation coefficient of approximately 20 S and

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