Alternative Pathways for Association and Dissociation of the Calmodulin-binding Domain of Plasma Membrane Ca\(^{2+}\)-ATPase Isoform 4b (PMCA4b)

Received for publication, May 1, 2012, and in revised form, June 26, 2012. Published, JBC Papers in Press, July 5, 2012, DOI 10.1074/jbc.M112.377556

John T. Penniston\(^1\), Ariel J. Caride\(^1\), and Emanuel E. Strehler\(^1,\(^2\)

From the \(^1\)Department of Neurosurgery, Massachusetts General Hospital, Boston, Massachusetts 02114 and the \(^2\)Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, Minnesota 55905

**Background:** Plasma membrane Ca\(^{2+}\)-ATPase (PMCA) pumps are regulated by calmodulin (CaM) binding to the cytosolic tail.

**Results:** The CaM C-terminal lobe associates first with Trp-1093, followed by N-terminal lobe wrapping around Phe-1110 in PMCA4b. During dissociation, Trp-1093 leaves CaM first, resulting in a long-lived intermediate.

**Conclusion:** Alternative pathways govern association and dissociation of Ca\(^{2+}\)-CaM and the PMCA4b CaM-binding sequence.

**Significance:** The long-lived dissociation intermediate may keep the PMCA primed for activation.

The calmodulin (CaM)-binding domain of isoform 4b of the plasma membrane Ca\(^{2+}\)-ATPase (PMCA) pump is represented by peptide C28. CaM binds to either PMCA or C28 by a mechanism in which the primary anchor residue Trp-1093 binds to the C-terminal lobe of the extended CaM molecule, followed by collapse of CaM with the N-terminal lobe binding to the secondary anchor Phe-1110 (Juranic, N., Atanasova, E., Filoteo, A. G., Macura, S., Prendergast, F. G., Penniston, J. T., and Strehler, E. E. (2010) J. Biol. Chem. 285, 4015–4024). This is a relatively rapid reaction, with an apparent half-time of \(-1\) s. The dissociation of CaM from PMCA4b or C28 is much slower, with an overall half-time of \(-10\) min. Using targeted molecular dynamics, we now show that dissociation of Ca\(^{2+}\)-CaM from C28 may occur by a pathway in which Trp-1093, although deeply embedded in a pocket in the C-terminal lobe of CaM, leaves first. The dissociation begins by relatively rapid release of Trp-1093, followed by very slow release of Phe-1110, removal of C28, and return of CaM to its conformation in the free state. Fluorescence measurements and molecular dynamics calculations concur in showing that this alternative pathway of release of the PMCA4b CaM-binding domain is quite different from that of binding. The intermediate of dissociation with exposed Trp-1093 has a long lifetime (minutes) and may keep the PMCA primed for activation.

The plasma membrane Ca\(^{2+}\)-ATPase (PMCA) pump constitutes the primary mechanism for the expulsion of Ca\(^{2+}\) from all eukaryotic cells (1). Mammalian cells express four different isoforms (PMCA1–4), each with multiple splice variants. Emerging evidence suggests that the PMCAs are responsible for setting the basal level of intracellular [Ca\(^{2+}\)] and for the spatiotemporal control of local Ca\(^{2+}\) signals (2). To fulfill their various roles in cellular Ca\(^{2+}\) handling, different PMCAs are tightly regulated. Arguably the most important regulator of PMCA activity is calmodulin (CaM), a Ca\(^{2+}\) sensor protein involved in many Ca\(^{2+}\)-dependent processes in the cell (3, 4). The mechanism of CaM regulation of the PMCA involves the release of an autoinhibitory domain upon binding of CaM to the C-terminal tail of the pump. The CaM-binding region in the C-terminal tail of PMCA4b is contained within a 28-residue peptide named C28 (5). Different PMCA isoforms vary in their affinity for CaM and in the kinetics of their activation by Ca\(^{2+}\) and CaM (6–8). This is of functional significance: PMCAs with fast kinetics (PMCA2b and PMCA3f) are expressed predominantly in cells that require rapid Ca\(^{2+}\) clearance after a spike, such as neurons, whereas the “slow” PMCA4b may be optimized to handle tonic Ca\(^{2+}\) loads in non-excitable cells (7, 9).

The structural basis for the different Ca\(^{2+}\) activation kinetics of PMCA isoforms is incompletely understood but is likely related to the dynamics of CaM binding and dissociation. The overall binding of Ca\(^{2+}\)-CaM to peptide C28 can be observed by following the fluorescence of 2-chloro-(amino-Lys-75)-[6-[4-(N,N-diethylamino)phenyl]-1,3,5-triazin-4-yl]CaM (TA-CaM) during the binding process (10). We have shown that this method reflects two distinct steps of binding of C28 to CaM. In the first step, C28 binds to the C-terminal lobe of CaM, and Trp-1093 is buried in a hydrophobic pocket of CaM. This is accompanied by a small rise in the fluorescence of TA-CaM. In the second step, the CaM wraps around C28, with the N-terminal lobe of CaM binding to the C terminus of C28 (11, 12). This second step is accompanied by a large decrease in the fluorescence of TA-CaM (13). The binding of C28 to CaM is very tight, so we would expect a rapid association and a slow dissociation. Kinetic measurements have confirmed this is the case, but the mechanism by which this is accomplished is unclear. Using a combination of fluorescence stopped-flow kinetic measure-
ments and molecular dynamics calculations based on the recently solved structure of the CaM-C28 complex (12), we now show that the preferred pathway of dissociation may not be a simple reversal of association but instead involves rapid release of the N-terminal anchor Trp-1093 from the C-terminal lobe of CaM, followed by the much slower dissociation of the C-terminal anchor Phe-1110. This biphasic dissociation mechanism allows for the existence of a stable intermediate complex in which CaM is bound only via its N-terminal lobe to the PMCA. The long half-life of this complex may keep PMCA4b “primed” for activation under conditions in which the free Ca^{2+}-CaM concentration is low.

**EXPERIMENTAL PROCEDURES**

**Reagents and Peptides—**CaM was from Calbiochem. TA-CaM was prepared as described (10, 14) and was a generous gift from Dr. Katalin Török (St. George Hospital Medical School, London, United Kingdom). Peptides C28 and C28W/F were synthesized in the Mayo Clinic Peptide Core Facility and purified by HPLC to at least 95% purity. All chemicals were of the highest purity available.

**Stopped-flow Kinetic Assays—**Stopped-flow measurements of changes in the fluorescence of TA-CaM were performed in an Applied Photophysics SX.18MV reaction analyzer. When measuring changes in the fluorescence of TA-CaM, the excitation wavelength was 365 nm, and the emitted light was detected using a 390-nm cutoff filter. In all cases, the syringes contained 30 mM TES/triethanolamine (pH 7.2), 120 mM KCl, 5 mM MgCl₂, 0.2 mM EGTA, and enough CaCl₂ to obtain 100 μM free Ca^{2+}.

**Association Rate Measurements—**To measure association following changes in Trp fluorescence, final concentrations were 2 μM C28 and either 2 or 4 μM CaM. In this case, excitation was set at 290 nm, and the emitted light was detected using a 345-nm cutoff long-pass filter. Because binding of C28 to CaM results in a shift in the Trp fluorescence to lower wavelength (blue shift), binding resulted in decreased light detected under these conditions (see Fig. 1). To assess the binding of C28 to TA-CaM, fluorescence changes were followed upon addition of C28 (at the concentrations indicated in Fig. 7) to TA-CaM (final concentration of 17 nM).

**Dissociation Rate Measurements—**To measure the dissociation of TA-CaM from C28, preincubated TA-CaM and C28 were mixed with unlabeled CaM. Final concentrations were 17 nM TA-CaM, 100 nM C28, and 2000 nM CaM. Because of the slow rate of this reaction, the best results were obtained when the change in fluorescence was followed in a Varian Cary Eclipse spectrofluorometer in kinetics mode rather than by stopped flow. Excitation was set at 365 nm, and the emission wavelength was set at 420 nm, with a 10-nm bandwidth. To measure the dissociation of C28 from CaM by Trp fluorescence, preincubated C28 and CaM were mixed with C28W/F, a peptide with the same sequence as C28 except for the replacement of the Trp residue at position 8 with Phe. Final concentrations were 2 μM C28, 4 μM CaM, and 40 μM C28W/F. Because of the fast nature of this reaction, it was followed by stopped flow using the conditions described above for measuring Trp fluorescence during association. Individual progress curves were fitted by nonlinear regression using GraphPad Prism 4 software. When global nonlinear regression was applied to fit a proposed model to progress curves obtained at different concentrations, DynaFit software (BioKin, Inc.) (15) was used.

**Molecular Dynamics (MD) Simulations—**MD calculations were performed using CHARMM c35b1 (16) running under Linux on the Partners Research Computing Facility in Boston. The coordinates for the CaM-C28 complex were those of the first structure from the set deposited in the Protein Data Bank (code 2KNE). CHARMM was run using the CHARMM22 all-hydrogen parameter and topology files for proteins, including φ and ψ cross-term map corrections. All minimization and dynamics were performed using the “generalized Born using molecular volume” method (17) to correct for solvation of the system. This method replaces water molecules with correction parameters that simulate the presence of water molecules. The parameters used were as follows: Beta = 20, Epsilon 80, Dn 1.0, Wartr 1.4, Geom Tol 1e−8, Buftr 0.5, Mem 10, Cuta 20, Hsx1 −0.125, Hsx2 0.25, Alfqr 1, Emp 1.5, P4 0.0, P6 8.0, P3 0.70, Onx 1.9, Offx 2.1, Wtyp 2, Nphi 38, shift −0.102, slope 0.9085, Corr 1, Ctonbb 12, Ctofnn 14, Cutnb 16, Vswitch, Switch.

**Targeted MD (TMD)—**Minimization was done with constraints gradually removed; for Protein Data Bank structure 2KNE, the final stage was 3000 steps of the adopted basis Newton–Raphson method with no constraints. Dynamics for structure 2KNE alone were begun by heating for 6 ps using a step size of 1 fs, the leapfrog integrator, a starting temperature of 200 K, and a final temperature of 310 K. Heating was done every 100 steps, with velocities scaled using a Gaussian distribution. Equilibration was done twice, first with Langevin dynamics for 20 ps, with the temperature bath at 310 K. Then the system was equilibrated for another 20 ps with constant pressure/temperature dynamics, with a reference temperature of 310 K. This double equilibration was found to produce a stable TMD run. The same 2KNE restart file was used for all of the TMD runs. Because a number of different target conformations were tested, it was necessary to prepare a minimized set of coordinates for each. The first structure determined by NMR for free Ca^{2+}-CaM (code 1X02) was visualized in Swiss-PdbViewer (18), and C28 in the appropriate conformation was manually added at a distance of ~200 Å from the CaM. This target structure was minimized with a final stage of 100 steps of the adopted Newton-Raphson method. It was not necessary to do any dynamics on these minimized structures because they were just used as targets. Production runs of TMD were performed for 30,000 steps using the same settings with constant pressure/temperature dynamics, with a reference temperature of 310 K and coordinates written every 1000 steps.

**Random Expulsion MD (REMD)—**For this method, the 2KNE structure was heated and equilibrated as described for the first equilibration of the TMD method, but it was not necessary to switch to constant pressure/temperature dynamics and the production runs were done using Langevin dynamics. Once the system was equilibrated, random number generators were used to select 1) a direction for pushing the C28 molecule and 2) three Ca atoms from adjoining residues to push upon. The Cα atoms for pushing were chosen to be between Ile-1091...
and Phe-1110. The force of pushing was set at 2 nanonewtons. After 20 steps of dynamics, the change in the root mean square deviation (r.m.s.d.) of the three Ca atoms from their neighbors in CaM was estimated. If this was <0.03 Å, a new set of Ca atoms and a new direction were chosen for the next round; otherwise, the next round of dynamics was on the same atoms and in the same direction as before. The run was continued until 2000 groups of 20 steps each had been run (total number of 40,000). Although the steps correspond to 2 fs, the presence of the pushing force in both TMD and REMD means that the system moves much faster than an actual system would, i.e. the progress time of these MD simulations cannot be directly compared with the time frame of the measured fluorescence changes in kinetic experiments. The governing factor in this procedure is the magnitude of the pushing force. If a smaller pushing force was used, more steps were required, but the results were essentially the same.

RESULTS

Kinetic Measurements of CaM and C28 Interaction Suggest Alternative Preferred Routes for Association and Dissociation—The fluorescence changes in TA-CaM during binding of C28 to CaM were reported previously and showed that the binding is moderately rapid and characterized by an initial fast fluorescence increase, followed by a slower two-phase decrease (13). Detailed kinetic analysis revealed that this could be explained by a three-step mechanism corresponding to a rapid initial association, followed by two steps stabilizing the complex (10, 13). Fig. 1 shows the Trp fluorescence changes during binding of C28 to CaM at two different CaM concentrations (2 and 4 µM). The Trp fluorescence reflects a different aspect of the reaction from that revealed by the TA-CaM fluorescence because it reports the change in the environment of the Trp residue on C28 (corresponding to Trp-1093) rather than of the fluorescence label on CaM. The observed decrease in fluorescence intensity showed two components, a fast one and a slower one. The rapid first step may represent the initial contact of Trp-1093 with the hydrophobic pocket in the C-terminal lobe of CaM, followed by a slightly slower stabilization in the pocket. Such a multistep interaction of CaM with its target peptide upon binding has previously been rationalized by Wand and co-workers (19) to reflect the initial formation of a low affinity complex, followed by conformational rearrangements resulting in the final high affinity complex. This result also fits well with the mechanism proposed earlier for the binding of C28 to CaM, in which Trp-1093 binds to the C-terminal lobe of CaM, followed by the wrapping of CaM around C28, with the N-terminal lobe of CaM binding to Phe-1110 (12). The rapid change in the Trp fluorescence reflects the initial binding of C28 to CaM, whereas the more complex change in TA-CaM fluorescence reflects all steps of the reaction.

The TA-CaM fluorescence during the dissociation of C28 is shown in Fig. 2A. As expected, the dissociation was very slow (20). Remarkably, however, the fluorescence of Trp during dissociation changed much more quickly than that of TA-CaM (Fig. 2B). The speed with which the fluorescence of Trp-1093 changed during the dissociation was unexpected because it indicated that the preferred dissociation process proceeds by a quite different pathway than association, resulting in a branched model (Fig. 2C) for the association/dissociation of CaM and C28. In particular, this result indicates that the extended intermediate with Trp-1093 embedded in a pocket in the C-terminal lobe of CaM (represented by Protein Data Bank NMR structure 1CFF) occurs preferentially during the initial binding of CaM to its target on PMCA, but may not be the major conformer during dissociation. We next turned to MD to simulate the dissociation process to better understand the possible mechanism underlying these findings.

MD Suggests a Directional Path for Dissociation of C28 from CaM—Analysis of the structure of the complex of CaM-C28 (Protein Data Bank code 2KNE) showed that C28 is much more accessible to solvent on one of its sides. Fig. 3 shows the complex oriented so that its top or open face is upward, facing the +z direction, and the bottom or closed face is down. In the complex, Trp-1093, with its large side chain, has more close interactions with CaM residues than does Phe-1110. The binding pockets of these two anchor residues were defined as the atoms within 6.0 Å of the side chain. Table 1 lists these atoms.

Because MD calculations are too slow to simulate the dissociation of C28, we first used TMD to simulate the process. Normally, this method is used when both the initial and final (target) conformations of the molecules are known. Starting from the initial conformation, a small force is applied in the direction of the target. This force is added to the normal force fields employed in MD calculations, allowing the molecule to reach its target conformation in a usable amount of calculation time. The presence of this force means that each MD step, which would normally correspond to 2 fs, now would correspond to a longer time. Good results have been obtained by this method in a number of cases (21, 22). In our case, we know the initial conformations of the CaM-C28 complex and the final free Ca2+-CaM, but the final conformation of C28 is not known. This is of little consequence, however, because we were primarily interested in whether the environment of Trp-1093 changed rapidly in the initial steps of the dissociation. Our results bore out this assumption about the conformation of C28, but we found that the placement of C28 in space influenced the results. When the target C28 was placed in the +z direction, opposite the open face of CaM-C28, Trp-1093 came out of its pocket long before Phe-1110, as shown in Fig. 4 (left panel). In this
panel, the increase in the average distance of Trp-1093 from the atoms making up its initial pocket is plotted as a function of the number of steps of MD. The conformation of C28 and its orientation were not important factors in these results. Helical and extended target C28 molecules were oriented parallel, antiparallel, and perpendicular to the starting C28 helix. In Fig. 4, all of these conformations and orientations are averaged together, with the small error bars reflecting the variations caused by the different runs.

Also shown in Fig. 4 is the r.m.s.d. for CaM as the system moves from its starting point toward the target. The targets were all set with the target CaM (Protein Data Bank code 1X02) oriented so that it had the minimum r.m.s.d. from the initial CaM structure in the C28 complex. This r.m.s.d. was 11.44 Å for all of the backbone atoms. As the targeted MD run proceeded, the r.m.s.d. of the CaM at any given time gradually increased; if it reached the target conformation, it would have a value of 11.44 Å. Thus, the r.m.s.d. shown in Fig. 4 is a measure of how closely CaM (not including C28) approached its target conformation during the run. In Fig. 4 (left panel), the changes in the CaM conformation shown by the r.m.s.d. are quite modest as Trp-1093 comes out of its pocket.

When the target C28 was placed below the starting CaM-C28 complex (in the $-z$ direction), Phe-1110 came out of its pocket before Trp-1093 (Fig. 4, right panel). However, in order for this to happen, CaM had to move about half-way toward its target conformation, as shown by its r.m.s.d. changes. Only after that had happened did Phe-1110 begin to leave its pocket, whereas Trp-1093 stayed near its original pocket atoms.

The experimental data show that Trp fluorescence changed much more rapidly than CaM conformation (as represented by TA-CaM fluorescence) during the dissociation of the complex. The results of TMD agree with this when the target C28 was placed in the $+z$ direction, but not when it was placed in the $-z$ direction. Thus, taken together, the results can only be explained if C28 actually leaves the CaM-C28 complex in the $+z$ direction.

REMD Shows That Trp-1093 Leaves CaM before Phe-1110 during Dissociation—Current TMD simulations are known to have a bias in that the larger movements in a transition path tend to happen early in the run and the smaller movements late (23). Because of this and to obtain a more consistent picture of how C28 dissociates, we turned to another variant of MD, REMD (24). Instead of adding a force directed toward a target, our method added a randomly oriented force, which pushed on three adjacent Ca atoms of C28 for a score of steps before the result was assessed. If this push resulted in substantial movement of C28, this was taken as an indication that movement in that direction was easy, and pushing continued in that direction until resistance was encountered. At this point, pushing was

![FIGURE 2. Kinetics of the dissociation of CaM and C28 and fitting to a branched model.](image)

**A**, time course of the dissociation of CaM from C28 as measured by TA-CaM fluorescence. Because of the very slow dissociation, the fluorescence change was measured in a Varian Cary Eclipse fluorometer as described under "Experimental Procedures." **B**, progress curve of the dissociation of CaM from C28 as determined by Trp-1093 fluorescence. **C**, branched model used to fit the data in **A** and **B**. The black lines in **A** and **B** are the fits using the rate constants listed in Table 2. The model proposes different CaM-C28 intermediate complexes (denoted as CaM-C28* and CaM-C28#) that are preferentially formed during association and dissociation, respectively.

![FIGURE 3. Structural model of the stable CaM-C28 complex (Protein Data Bank code 2KNE).](image)

CaM is shown in dark gray, with its N- and C-terminal lobes labeled. Peptide C28 is in light gray; the anchor residues Trp-1093 and Phe-1110 (medium gray), indicated by arrows, are difficult to see because they are nearly buried in other residues. Note how the N-terminal region of C28 is available to solvent, whereas the C-terminal half is much more buried in the CaM N-terminal lobe.
tried in another randomly chosen direction on another randomly chosen part of C28. This method explores the various ways in which C28 could leave CaM without utilizing an arbitrary target. Fig. 5 shows the average of 52 REMD runs; this average clearly shows Trp-1093 leaving its pocket first, with Phe-1110 leaving later. The r.m.s.d. of CaM compared with its starting point changed much more slowly than that of the anchors leaving their pockets. This is especially significant because the r.m.s.d. of free CaM compared with CaM in the CaM-C28 complex is 11.44 Å.

Structurally Distinct States Characterize the Binding and Dissociation of PMCA4b and CaM—The above data suggest that the preferred binding and dissociation pathways for Ca\(^{2+}\)/CaM and C28 may differ, resulting in a branched (“diamond”) scheme as illustrated in Fig. 2C. Fig. 6 shows the different structural conformers that characterize these two branches. The structure at the top in Fig. 6 is the NMR structure of Ca\(^{2+}\)/H11001-CaM not bound to any peptide (Protein Data Bank code 1X02). This is an extended structure in which the two Ca\(^{2+}\)-binding lobes are separated by a central helix. The C-terminal half of CaM is

| 1 | 10 | 20 | 30 | 40 |
|---|---|---|---|---|
| ADQLTEEQI | AEFKEAFSLF | DKGDGTITTT | KELGTVRSL | GQNPTEAEQL |
| 50 | 60 | 70 | 80 | 90 |
| DMINEVDADG | NGTIDFPEFL | TMMARKMKDT | DSEEEREAFF | RVDKDGNGY |
| 100 | 110 | 120 | 130 | 140 |
| IAAELRHVM | TNLGEKLTDE | EVDMIEAR | IDGDGQVNYE | EFVQMMMTAK |

Binding pocket for Trp-1093 (total of 12 residues and 52 atoms)

| Phe-92 | Ile-100 | Leu-105 | Glu-123 | Met-124 | Ile-125 | Glu-127 | Ala-128 | Val-136 | Phe-141 | Met-144 | Met-145 |
|--------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| Cy-C81-Ce1-Cf1-C82-Ce2 | Cy2-C8 | Cβ-Cy-C81-C82 | C-O | N-Ca-Cβ-Cγ-Sβ-Cε-C-O | N-Ca-C-O | C | N-Ca-Cβ-C | Cβ-Cy1-Cy2 | Ca-Cβ-Cγ-C81-Ce1-Cf1-C82-Cε-C-O | Ca-Cβ-Cγ-Sβ-Cε-C-O | N |

Binding pocket for Phe-1110 (total of 9 residues and 35 atoms)

| Phe-19 | Ile-27 | Leu-32 | Val-35 | Met-36 | Met-51 | Ile-63 | Phe-68 | Met-71 |
|--------|---------|---------|---------|---------|---------|---------|---------|---------|
| Cy-Ce1-Cf1-C82-Ce2 | Cβ-Cy2-Cy1-C8 | Ca-Cβ-Cγ-C81-C82-C-O | Cβ-Cy1-Cy2 | Cy-Sβ | Cy-Sβ-Cε | Cβ-Cy2-Cy1-C8 | Cy-Cγ-C82-Ce2 | Cy-Sβ-Cε |
the right in Fig. 6 is the NMR structure of the complex of CaM with C20 (code 1CFF). C20 is depicted as a pale yellow ribbon, with Trp-1093 shown in space-filling mode in purple. Because a structure with very similar properties occurs at the first stage of C28 binding, in Fig. 6, the known structure of C20 was supplemented by adding the eight additional residues contained in C28, with Phe-1110 shown in space-filling mode in green. The structure shown at the bottom in Fig. 6 is the NMR structure of CaM with C28 (code 2KNE), and the structure shown on the left is one of the models from the targeted MD, in which C28 was in the +z direction.

Analysis of Kinetic Data Using the Branched Model for Association/Dissociation of CaM and C28—In our previous work, the association and dissociation kinetics of C28 and TA-CaM were modeled using a linear multistep model (13). In view of the above data, we refitted the kinetic results with the branched model indicated in Fig. 2C. The association of CaM with C28 may occur preferentially through one branch of the scheme, whereas the dissociation would occur preferentially through the other branch. To obtain the rate constants, we first fitted the model to an association experiment (Fig. 7) similar to that reported previously (13), in which TA-CaM was mixed with C28. We used the rate constants from that fit to refit the model to the results of TA-CaM dissociation as shown in Fig. 2A. The dissociation rate constants \( k_{-3} \) and \( k_{-4} \) and the quantum yield of the species were allowed to change, but the rest of the constants were fixed. This was done because dissociation rate constants are more likely to be accurately obtained from a dissociation experiment rather than from an association experiment. With the new values for \( k_{-3} \) and \( k_{-4} \), we refitted the association experiment (Fig. 7), this time allowing only the quantum yields to be varied. The results of the experiment measuring the change in Trp fluorescence during the dissociation of C28 from CaM were also fitted with this set of constants (Fig. 2B). This exercise was done to show that it is plausible to fit the three experiments with the same set of rate constants. The rate constants obtained as described are shown in Table 2. When comparing \( k_1 \) and \( k_3 \), their values are not that different. However, \( k_2 \) is twice as large as \( k_4 \), and in contrast, \( k_{-1} \) is three times smaller than \( k_{-3} \). This indicates that the complex formed by the route of binding Trp-1093 first and Phe-1110 in a second step is much more likely to result in a final complex than if Phe-1110 binds first. When looking at the dissociation reaction, \( k_{-3} \) is 5 orders of magnitude smaller than \( k_{-4} \), clearly indicating that the route in which Trp-1093 dissociates first is much more likely than the route in which Phe-1110 dissociates first. Thus, it is likely that the CaM-C28 complex forms by a route in which the C-terminal lobe of CaM binds first to the Trp-1093 anchor and the N-terminal lobe of CaM binds in a second step to Phe-1110, but that the dissociation of this complex prefers a route in which Trp-1093 dissociates first (as suggested by the structure on the left in Fig. 6).

**DISCUSSION**

The binding and dissociation mechanism depicted in Fig. 6 indicates that the PMCA exists in at least three activated forms while it is bound to Ca\(^{2+}\)-CaM. The first step in the binding of CaM results in the structure shown on the right in Fig. 6. This

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**FIGURE 5.** REMD of the dissociation of C28 from CaM. The data show that Trp-1093 leaves its binding pocket more rapidly than does Phe-1110, with very little change in conformation of CaM. In the Trp and Phe plots, the middle line represents the average distance from the pocket, and the flanking lines show S.E. The distance from the pocket was defined as described in the legend to Fig. 4. The results were averaged from 52 REMD runs.

**FIGURE 6.** Scheme of association/dissociation pathways of C28 and CaM and relevant structures. Structural models of the molecules at different stages are shown. The three for which Protein Data Bank codes are shown are based on the actual NMR structures; the fourth structure is an MD simulation. The N- and C-terminal lobes of CaM are colored light blue and pink, respectively; the Phe- and Trp-binding pockets are highlighted in blue and red, respectively. The C28 peptide is shown in yellow, and the important anchor residues Trp-1093 and Phe-1110 are shown in space-filling mode in purple and green, respectively. Thick arrows indicate the preferred structural transitions. Note that this is a structural illustration of the results of this study and not a kinetic diagram.

shown as a pale pink ribbon structure, with the residues of the Trp-binding pocket shown in space-filling mode in red. The N-terminal half of CaM is a pale blue ribbon, with the Phe-binding pocket shown in space-filling mode in blue. Shown on
structure corresponds to the highly fluorescent form of TA-CaM, which occurs in the early stages of binding to either C28 (13) or PMCA4b (10). We have shown here that the Trp fluorescence is decreased in this structure (Fig. 1). Detailed analysis of the kinetics of PMCA activation (10) showed that this form is already activated, in agreement with earlier findings that binding of the C-terminal lobe of CaM alone can activate the pump (11, 25). The second activated form is the stable CaM-PMCA complex represented by the structure at the bottom in Fig. 6. This corresponds to the structure in which TA-CaM fluorescence is quenched, which occurs in the later stages of binding to C28 or PMCA (see Fig. 7) (10). The third activated form is the intermediate we reported in this study, which occurs during dissociation and in which Trp-1093 leaves its pocket, but the CaM remains bound. This intermediate still has its TA-CaM fluorescence quenched, and the recovery of the fluorescence during dissociation is very slow (Fig. 2A). This correlates well with the inactivation of PMCA4b during the dissociation of CaM, which is also very slow (20).

Of the activated forms, the intermediate on the right in Fig. 6 (Protein Data Bank code 1CFF), which occurs preferentially during binding, is short-lived and exists only for a second or so before the collapse of CaM around its binding domain. The form at the bottom (code 2KNE) is stable, whereas the intermediate on the left may persist for many minutes. The prolonged exposure of Trp-1093 in this last form may have a biological function that we do not yet understand. However, the extended lifetime of this dissociation intermediate suggests that reactivation of the pump may be facilitated. This could increase the efficiency of PMCA4b under conditions of limiting CaM (but sustained elevated free Ca\(^{2+}\)) concentrations, where the complete dissociation of CaM might result in prolonged inactivity of the pump.

The results summarized schematically in Fig. 6 indicate that activation and inactivation of PMCA4b by Ca\(^{2+}\)-CaM occur by a different mechanism than has been assumed. We showed in our previous studies (10, 12) and confirmed here that the interaction with the C-terminal lobe of CaM (involving the Trp-1093 anchor) is a transitory intermediate in activation, and we have now described another, long-lasting intermediate that occurs during the preferred pathway of CaM dissociation. In this intermediate, Trp-1093 disengages from the C-terminal lobe of CaM first, contrary to previous models for the dissociation mechanism (26) that assumed that the N-terminal lobe of CaM releases the peptide first. Trp-1093 and Phe-1110 are usually referred to as “anchors” of the CaM-C28 complex. The experimental evidence and MD simulations shown here indicate that Phe-1110 can be the anchor provided by C28 in the Ca\(^{2+}\)-CaM-C28 complex. This is in agreement with our previous result (13) that showed that Phe-1110 was essential for the stabilization of the C28-CaM complex. It must be noted, however, that our results apply strictly only to a situation in which CaM remains Ca\(^{2+}\)-saturated during the association/dissociation process. Stopped-flow studies of other complexes of CaM with CaM-binding peptides have investigated dissociation induced by Ca\(^{2+}\) chelation with EGTA (27, 28), which is different from the experimental approach in this study. In those studies, the N-terminal lobe of CaM was shown to leave the C-ter-

![FIGURE 7. Time course of the association of CaM and C28 as measured by TA-CaM fluorescence and fitting to the branched model. C28 at the indicated concentrations was mixed with TA-CaM (final concentration of 17 nM). Conditions were as described under "Experimental Procedures." The colored lines are the fits using the branched model in Fig. 2C and the rate constants listed in Table 2. The inset shows the data and fits during the initial 0.2 s at an enlarged time scale for better resolution.](image-url)

| Rate constant | Value           |
|--------------|-----------------|
| $k_1$        | $0.0978 \text{ M}^{-1} \text{s}^{-1}$ |
| $k_{-1}$     | $3.42 \text{ s}^{-1}$ |
| $k_2$        | $1.93 \text{ s}^{-1}$ |
| $k_{-2}$     | $1.687 \times 10^{-8} \text{ s}^{-1}$ |
| $k_3$        | $0.167 \text{ M}^{-1} \text{s}^{-1}$ |
| $k_{-3}$     | $11.94 \text{ s}^{-1}$ |
| $k_4$        | $0.808 \text{ s}^{-1}$ |
| $k_{-4}$     | $0.001235 \text{ s}^{-1}$ |
minal of the peptides first, but this appeared to be related to the dissociation of Ca\(^{2+}\) from the N-terminal lobe of CaM rather than to the peptide anchor residue leaving CaM. More relevant to the study presented here, Toırök and Trentham (14) showed that the dissociation of TA-CaM from a peptide derived from myosin light chain kinase was slow, but it was faster if the association was interrupted before CaM collapse occurred. This result is in agreement with the idea that CaM dissociates faster when the complex involves only one anchor, but does not indicate which anchor is the one that is rate-limiting for slow dissociation. The results in this work, from both kinetic experiments and MD simulations, suggest that when CaM is loaded with Ca\(^{2+}\), Phe-1110, rather than Trp-1093, fulfills this function in PMCA4b. Which residue is the anchor under prevailing cellular conditions remains to be determined. Recent work using low drift atomic force microscopy has shown the feasibility of determining the unfolding/fragmenting and ligand association/dissociation landscape of CaM at the single-molecule level (29, 30). Single-molecule force spectroscopy may thus be a promising tool to experimentally test the proposed pathways of CaM-C28-target peptide association/dissociation predicted from fluorescence kinetics and TMD simulations.

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