Calmodulin Wraps around Its Binding Domain in the Plasma Membrane Ca\(^{2+}\) Pump Anchored by a Novel 18-1 Motif*\(\dagger\)

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Using solution NMR spectroscopy, we obtained the structure of Ca\(^{2+}\)-calmodulin (holoCaM) in complex with peptide C28 from the binding domain of the plasma membrane Ca\(^{2+}\)-ATPase (PMCA) pump isoform 4b. This provides the first atomic resolution insight into the binding mode of holoCaM to the full-length binding domain of PMCA. Structural comparison of the previously determined holoCaM-C20 complex with this holoCaM-C28 complex supports the idea that the initial binding step is represented by (holoCaM-C20) and the final bound complex by (holoCaM-C28). This affirms the existing multi-step kinetic model of PMCA4b activation by CaM. The complex exhibits a new binding motif in which holoCaM is wrapped around helical C28 peptide using two anchoring residues from the peptide at relative positions 18 and 1. The anchors correspond to Phe-1110 and Trp-1093, respectively, in full-length PMCA4b, and the peptide and CaM are oriented in an anti-parallel manner. This is a greater sequence distance between anchors than in any of the known holoCaM complexes with a helical peptide. Analysis of the geometry of holoCaM-peptide binding for the cases where the target peptide adopts an \(\alpha\)\(_D\)-helix with its anchors buried in the main hydrophobic pockets of the two CaM lobes establishes that only relative sequential positions of 10, 14, 17, and 18 are allowed for the second anchor.

Plasma membrane Ca\(^{2+}\)-ATPases (PMCA)\(^3\) are crucial components of Ca\(^{2+}\) regulation in eukaryotic cells. In mammals, four isoforms (PMCA1–4) and numerous splice variants are involved in the maintenance of overall intracellular Ca\(^{2+}\) homeostasis and in local Ca\(^{2+}\) signaling (1). The PMCAs are tightly regulated by multiple mechanisms including oligomerization, interaction with phospholipids, phosphorylation by Ser/Thr and Tyr kinases, and activation by Ca\(^{2+}\)-calmodulin (CaM) (2–4). Interaction with CaM is a distinguishing feature and arguably one of the most important mechanisms for PMCA activation.

CaM regulates the PMCA by interacting in a Ca\(^{2+}\)-dependent manner with a specific binding domain (CBD) in the C-terminal tail (C-tail) of the pump. In the absence of CaM, the PMCA activity is low because of autoinhibitory intramolecular interactions between the C-tail and the catalytic domain (Fig. 1). Autoinhibition is relieved by binding of CaM to the CBD, resulting in a structural transition accompanied by the formation of the CaM-CBD complex. Binding of Ca\(^{2+}\)-loaded CaM (holoCaM) to the CBD has been studied using peptides comprising different portions of the CBD (Fig. 1) as well as full-length and C-terminally truncated PMCAs expressed in mammalian or insect cells (5–9). Most of this work has been performed with the human isoform PMCA4 because this pump can be readily purified from erythrocytes and shows tight regulation by CaM.

Extensive kinetic studies with the peptides and full-length and mutant proteins combined with limited structural work using the short peptides provided essential information on the mechanism of CaM binding to the PMCA. A crucial structural difference in the binding of peptide C20 versus the longer C24 peptide was established early on by Kataoka et al. (8) using small angle x-ray scattering in liquids. Although the holoCaM-C20 complex remained in an extended conformation with a shape similar to that of free holoCaM, the holoCaM-C24 complex showed a much more compact structure. This led to the suggestion that the C20 mode of binding may represent the first step of CaM binding to the PMCA. This was further substantiated by an atomic level structure of the holoCaM-C20 complex determined using NMR by Elshorst et al. (7). An extended structure was found in which C20 binds only to the C-terminal lobe of CaM, with Trp-1093 (at position 8 of C20) as the most prominent anchoring residue. This finding was discussed in terms of the observation that the isolated C-terminal lobe of CaM can activate the PMCA, albeit with 100-fold less potency than intact holoCaM (5). Based on these data, the holoCaM-C20 complex was proposed to reflect the structural form by which holoCaM activates PMCA. However, a more detailed study of binding of the separated CaM lobes showed that the N-terminal...
Structure of the Calmodulin-PMCA C28 Peptide Complex

We report here the NMR solution structure of the *holo* CaM-C28 complex, thereby providing the first atomic resolution insight into the binding mode of *holo* CaM to the full-length CBD of PMCA4b. The available structures allow a comparison between the initial binding step (*holo*CaM-C20) and the final bound complex (*holo*CaM-C28) and suggest that these structural states correlate with the corresponding kinetic intermediates. We further evaluate how the new type of peptide-to-*holo*CaM anchoring in the CaM-C28 complex advances our understanding of target peptide recognition by *holo*CaM (11–13).

**EXPERIMENTAL PROCEDURES**

*CaM Expression and Purification—*Human CaM cDNA was subcloned into the pET-15b expression vector (Novagen, San Diego, CA) using standard molecular biology cloning techniques. No purification tag was introduced into the construct. The identity of the insert was confirmed by DNA sequencing. CaM was expressed in *Escherichia coli* BL21(DE3)-pLysS (Novagen, San Diego, CA) in LB rich medium containing the appropriate antibiotic. Uniform $^{13}$C/$^{15}$N enrichment of CaM was achieved by growing cells in LB medium and then inducing in minimal medium containing $[^{13}]C$-glucose and $^{15}NH_4Cl$, as single source of carbon and nitrogen, following established protocols (14). CaM was purified using a slight modification to an existing procedure (15). Upon addition of 5 mM CaCl$_2$, the cleared cell lysate was loaded onto a phenyl-Sepharose CL-4B column (Sigma), and CaM was eluted with 1 mM EGTA. The CaM fractions were further purified using a HiTrap Q column (Amersham Biosciences) and a 0–1 M NaCl gradient. CaM purity was greater than 95% as judged by SDS gel electrophoresis and Coomassie Brilliant Blue staining. The identity of the purified CaM was confirmed by N-terminal sequencing and by liquid chromatography-mass spectrometry analysis of its tryptic digest fragments. The molecular weight and labeling efficiency (96%) was verified with electrospray ionization-mass spectrometry by direct infusion. The final CaM buffer was 10 mM TES-TEA, pH 7.5, 100 mM KCl, 10 mM CaCl$_2$, 1 mM EDTA.

Expression and Purification of $^{13}$C/$^{15}$N Uniformly Labeled C28 Peptide—The calmodulin-binding peptide (C28W) from PMCA4b was expressed as a fusion protein with immunoglobulin-binding domain B1 of streptococcal protein G (GB1). The GB1-His$_6$-TEV-C28W cDNA was *de novo* synthesized (Blue-Heron Biotechnology, Bothell, WA), adding a codon for methionine to allow the release of the intact peptide after CNBr cleavage. The DNA construct was subcloned into the pET-15b expression vector (Novagen). Fusion protein expression was carried out in *E. coli* BL21(DE3) in minimal medium containing $[^{13}]C$-glucose and $^{15}NH_4Cl$ as single source of carbon and nitrogen, respectively. After expression, the cells were pelleted and resuspended in 8 mM urea, 50 mM phosphate buffer, pH 7.7, containing 600 mM NaCl, and the lysate was loaded on a HisTrap FF column (GE Healthcare). Refolding was performed on the column by decreasing the urea concentration in the buffers. The fusion protein was eluted with 250 mM imidazole, lyophilized to dryness, desalted on a G25 Sephadex column (10 mM NH$_4$HCO$_3$ at pH 8.0), and lyophilized again. CNBr cleavage was performed in 0.1 M HCl with the addition of CNBr at a molar ratio of 100:1 of CNBr to substrate for 18 h at 16 °C in the dark. The solution was then diluted 10-fold with distilled deionized H$_2$O and lyophilized to dryness. The samples (dissolved in distilled deionized H$_2$O) were subjected to reverse phase HPLC (Beckman Coulter) using a C$_{18}$ column (Jupiter 5 μm C$_{18}$; Phenomenex), and individual peaks were collected. The quality and the molecular weight of the peptide were assessed and verified by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy. Peaks containing pure peptide were pooled and twice lyophilized to dryness to remove all organic solvents. The labeling efficiency (98%) was verified with electrospray ionization-mass spectrometry by direct infusion. The peptide was dissolved in a final buffer of 10 mM TES-TEA, pH 7.5, 100 mM KCl, 10 mM CaCl$_2$, 1 mM EDTA.
CaM·C28W Complex Formation—The CaM-$^{13}$C, $^{15}$N-C28W complex was formed in the above buffer at 4 °C. The concentration of the peptide was kept constant while increasing the amount of CaM. The mixture was incubated for 1 h at 4 °C. Complex formation was monitored by native gel electrophoresis and Coomassie Blue staining. The same procedure was used for $^{13}$C, $^{15}$N-CaM·C28W complex formation except that in this case the concentration of $^{13}$C/$^{15}$N-CaM was kept constant while increasing the amount of C28W peptide.

Samples for NMR Spectroscopy—Four samples were prepared, all containing 2 mM holoCaM·C28 complex in aqueous (5% D$_2$O) solution, 10 mM TES-TEA, 100 mM KCl, 10 mM CaCl$_2$, 1 mM EDTA at pH 7.5. Sample 1 was uniformly labeled in CaM ($^{13}$C, $^{15}$N-CaM·C28W complex). Sample 2 was uniformly labeled in C28 (CaM-$^{13}$C, $^{15}$N-C28W complex). Sample 3 was the same as Sample 1, and Sample 4 was the same as Sample 2, except that orienting medium (Pf1 magnetic resonance cosolvent; Asla Biotech Ltd, Riga, Latvia) was added in a concentration to achieve anisotropy splitting of the solvent $^2$H signal of $\sim$16 Hz.

NMR Spectroscopy—NMR experiments were performed at 25 °C on a 700-MHz spectrometer (Bruker Avance 700) equipped with cryo-probe. Resonance assignments were obtained by using a combination of standard triple resonance experiments (16). Two-dimensional HNCA, HN(CO)CA, HNCACB, and CBCA(CO)NH were used for the backbone $^1$H, $^{15}$N, and $^{13}$C assignments and three-dimensional HAHB(CO)NH, (H)CC(CO)NH, HCCH COSY, and HCCH total correlation spectroscopy spectra, whereas the sequential assignment was cross-checked for consistency with three-dimensional $^{15}$N-edited HCCH COSY and HCCH total correlation spectroscopy spectra, whereas the sequential assignment was based on NOE connectivity in the $^{13}$C-edited ($^1$H,$^1$H) NOESY spectrum. Methionine methyl-group $^1$H and $^{13}$C assignment was achieved using methionine-methyl $^{13}$C$_e$-filtered ($^1$H,$^1$C) heteronuclear single quantum coherence and $^{13}$C$_{ve}$-filtered ($^1$H,$^1$C,$^{13}$C) HMBC spectra (17). NOESY spectra for structural constraints, $^{13}$C-edited ($^1$H,$^3$H) three-dimensional NOESY and $^{15}$N-edited ($^1$H,$^3$H) three-dimensional NOESY spectra were recorded at mixing times of 50 and 100 ms. The peptide-protein cross-peaks were identified by $^{13}$C, $^{15}$N-half-filtered, $^{13}$C, $^{15}$N-edited ($^1$H,$^3$H) three-dimensional NOESY spectra (18). A quantitative correlation HNHA experiment (19) was used for measurement of $^3$J($^3$H$^4$N$^5$) couplings. Residual dipolar couplings (RDC) were measured using IPAP-J-TROSY HNCO experiments (20) on Samples 3 and 4. The heteronuclear ($^1$H,$^1$N,$^{13}$N) NOESY was measured at 700 MHz.

The multidimensional spectra were processed and analyzed in FELIX (FELIX 2007-Felix NMR Inc.). All post pick-picking analyses (assignments and structural parameter calculations) were done using home-written programs in MATLAB (The MathWorks, Inc.; R2007a). $\sim$2500 NOE distances (including 114 peptide-protein distances) were extracted (see Table 1) and applied with error limits deduced from the cross-peak intensities. Protein backbone RDCs were determined for the almost complete set of $^1$D$_{NH}$ (139 of 144 for CaM and 26 of 27 for C28; the C28 Trp side chain $^1$D$_{NH}$ was also included in this set), and $\sim$70% of $^1$D$_{CC}$ and $^1$D$_{CN}$, all applied with an error limit of 0.5 Hz. The $^3$J($^3$H$^4$N$^5$) couplings were determined for $\sim$90% of the residues (128 CaM and 25 C28) and were applied with an error limit of 2 Hz.

Backbone H-bonds were assigned to all $\alpha$-helix and $\beta$-sheet elements of secondary structure, as identified by NOE contacts and $^\alpha$ chemical shift index (63 for CaM and 17 for C28) and applied as distance constraints. Each H-bond was characterized by two distance restraints $r_{N\cdots O} = 2.3$ with limits 2.0–3.0 Å and $r_{N\cdots O} = 3.3$ with limits 3.0–4.0 Å. Additional distance constraints were defined to maintain coordination geometry around Ca$^{2+}$ ions (21). Dihedral angle constraints for backbone PHI and PSI torsions were determined from backbone chemical shifts (259 for CaM and 42 for C28) using the program PREDITOR (22) and applied with limits of $\pm$ 30 deg. Calculation of various structural parameters (distances, angles, and dihedrals) from atomic coordinates in the Protein Data Bank structures was performed in MATLAB using home-written programs.

Structure Calculation—The structures were calculated on a Linux work station using simulated annealing with RDC protocols in Xplor-NIH (version 2.21). The best 20 conformers from 100 simulated annealing structures were selected on the basis of XPLOR energy, which included bond, angle, dihedral, van der Waals’, improper, Ramachandran, NOE, RDC (SANI), J coupling, and H-bond energy terms.

Data Deposition—The assigned chemical shift values, constraints, and atomic coordinates for the conformers were deposited at BioMagResBank (entry ID 16465) and Protein Data Bank (entry 2kne).

RESULTS AND DISCUSSION

NMR Solution Structure of Ca$^{2+}$-CaM·C28—Chemical shift perturbations (at the holoCaM backbone) upon C28 binding indicate that the entire CaM molecule is affected by the binding (Fig. 2A). A comparison with the holoCaM shifts induced by two other peptides, C20 and M13, is shown in Fig. 2B. CaM wraps around peptide M13 (CBD peptide from myosin light chain kinase) with both lobes contacting the peptide (21), which is different from the case of peptide C20 binding to the CaM C terminus only (7). As seen in Fig. 2B, binding of C28 affects the chemical shifts in both lobes of CaM, as observed for M13, but not for C20.

We used protein backbone RDCs for construction of an initial structure of the holoCaM·C28 complex (12, 23). A good match (correlation coefficient $r^2 = 0.9$ between calculated and experimental RDCs) for the CaM C-terminal lobe was found in the x-ray structure of a complex of holoCaM with the CBD of CaM-dependent protein kinase I (Protein Data Bank entry 1mxw (24)) and for the N-terminal lobe in the x-ray structure of free holoCaM (Protein Data Bank entry 1exr (25)). The optimized relative orientation of the two lobes was consistent with a rigid conformation found in structures having CaM wrapped around a helical peptide (12, 23). In support of this, RDCs from the whole CaM (both lobes) have the same “powder pattern” profile as RDCs from a single lobe (Fig. 3), indicating a rigid arrangement of the two lobes.
Peptide C28 exhibits RDC values close to those of an ideal \( \alpha \)-helix (Fig. 4A); the peptide is also rigid over most of its length as evidenced by \( ^1\text{H}, ^{15}\text{N} \) heteronuclear NOEs (Fig. 4B). For the first two residues of C28, the backbone amide resonances were not observed, indicating they are not part of the peptide helical structure. Because CaM appears to be wrapped around the C28 peptide, one would expect anchoring of some of the C28 side chains in the hydrophobic pockets of the two CaM lobes. Indeed, the Trp side chain from C28 (corresponding to Trp-1093 in full-length PMCA4b) was readily identified as the major anchoring residue to the C-terminal hydrophobic pocket of CaM. The stand-alone chemical shift of \( ^{1}\text{H}, ^{15}\text{N} \) TROSY spectrum with the residue numbers according to their position in PMCA4b (the Trp-1093-H\(^+\) signal is from the Trp-1093 side chain indole).

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Identification of a possible anchoring residue(s) to the N-terminal hydrophobic pocket of CaM proved more difficult. By
TABLE 1

Statistics for the ensemble of 20 structural models of the holoCaM-C28 complex

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**holoCaM-C28 (CaM, C28)**

| Experiment | Unit |
|------------|------|
| NOE distance constraints | 1113 (881, 232) |
| Intrasite (| 1000 | 1000 |
| Sequential (| 664 | 535 | 129 | |
| Medium range (| 355 | 262 | 93 | |
| Long range (| 201 | 100 | 0 | |
| Protein-peptide | 114 |
| Total NOE | 2447 |
| Torsion angle constraints (θ, ϕ) | 303 | 261 | 42 | |
| RDC constraints (\(D_{NCaM} \), \(D_{CNN} \)) | 376 | 327 | 49 | |
| N, K, L coupling constraints | 139 | 114 | 25 | |
| H-bonds | 80 | 63 | 17 | |
| RMSD from distance constraints (Å) | 0.05 ± 0.005 |
| RMSD from dihedral angle constraints (deg.) | 0.18 ± 0.05 |
| RMSD from RDC constraints (Hz) | 0.60 ± 0.05 |

**XPLOR energies (kcal/mol)**

- Total: -300 ± 40
- Van der Waals: 88 ± 6

**Ramachandran plot statistics (%)**

- Residues in most favored region: 89.2
- Residues in additionally allowed regions: 10.2
- Residues in generously allowed regions: 0.6
- Residues in disallowed regions: 0

**Coordinate precision (Å)**

- Backbone atoms: 0.60
- All atoms: 1.26

**RMSD from idealized covalent geometry**

- Bonds: 0.014 ± 0.003
- Angles: 1.6 ± 0.01

* RMSD between ensemble of 20 structures and the mean structure.

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Comparing \(^{13}\text{C},^{15}\text{N}\) fully and half-filtered two-dimensional \(^{1}H,^{2}H\) NOESY spectra obtained from the complex with \(^{13}\text{C},^{15}\text{N}\)-labeled CaM, strong CaM-C28 contacts were indicated for the aromatic residues of C28 (supplemental Fig. S1). However, resonances from the side chains of Trp-1093, Phe-1094, Phe-1110, and His-1111 overlapped in the region of interest, making it difficult to resolve whether there was an additional anchor at the C-terminal end of the C28 sequence. To overcome this problem, we recorded the \(^{13}\text{C}\)-edited three-dimensional \(^{1}H,^{2}H\) NOESY spectrum of \(^{13}\text{C},^{15}\text{N}\)-labeled C28 in the complex with unlabeled CaM (supplemental Fig. S1). Using this strategy, we were able to identify Phe-1110 (residue 25 of C28) as the major anchor to the N-terminal hydrophobic pocket in CaM. This finding reveals a novel way in which CaM wraps around a helical peptide, utilizing an 18-1 type of anchoring.

With two anchors known and with the relative orientation of three structural motifs (two EF hand lobes of CaM and the α-helix of C28) constrained by RDCs, the initial structure was constructed by positioning these motifs as rigid bodies. This structure was then subjected to simulated annealing to fulfill ~3,500 constraints (as specified in Table 1). The final structure is reported as an assembly of 20 conformers (statistics of the ensemble are given in Table 1, structural features are presented in Fig. 5, and the correlation between the experimental and calculated \(\Delta_{HN} \) for the representative conformer is illustrated in supplemental Fig. S2). As shown in Fig. 5, the backbone structure of holoCaM-C28 is very well defined in a rigid wrap-around conformation (ensemble RMSD of heavy atoms, 0.60 Å). The interlobe linker region has an only slightly less defined conformation. The C28 peptide is helical in region 4–26 with some distortion from the ideal α\(_h\) conformation. A comparison with the structures of other Ca\(^{2+}\)-CaM-peptide complexes where a peptide Trp serves as anchor to the C-terminal domain and a Phe serves as anchor to the N-terminal domain of CaM is given in Table 2. The closest structural neighbor to the novel holoCaM-C28 type anchoring appears to be the complex of holoCaM with the ryanodine receptor peptide (26), which has 17-1 anchoring (Table 2, Protein Data Bank entry 2bxc). There are no structural examples of 16-1 and 15-1 anchoring, but 14-1 is represented with at least two structures (Table 2). The CaM lobes and the anchoring site structures are very well conserved in this series of complexes. For the backbone atoms, the N- and C-terminal domains of holoCaM in the C28 complex have RMSD values of ~0.8 and 0.7 Å, respectively, from the CaM-peptide x-ray structures with Protein Data Bank codes 2bxc and 2fot (Table 2). The Trp anchor site has an RMSD value of ~0.7 Å, and the Phe site has an RMSD value of ~1 Å for the heavy atoms from the same x-ray structures. Therefore, the holoCaM-C28 structural features are very similar to those of other complexes in this series except for the relative positioning of the lobes in the novel 18-1 anchoring.

Novelty of 18-1 Anchoring of Peptide to CaM—As shown in Table 2, Phe-Trp anchoring of a helical peptide to holoCaM in an anti-parallel manner has been reported in several structures. We analyzed the structural relationships in the Phe-Trp anchoring family of holoCaM-peptide complexes and found them to be very consistent. In all cases the peptide is fully helical in the region between the two anchors, and the aromatic rings of the anchors are buried inside the CaM lobes in such a way that they point toward the β-sheet contact of the Ca\(^{2+}\)-binding sites. To fulfill such constraints, the two lobes of CaM must be rotated with respect to each other as represented in Fig. 6. In going from 18-1 to 17-1 anchoring, the helical distance (\(L \)) between the two anchors is shortened by the helical pitch distance, and the N-terminal lobe has to rotate clockwise for 360\(\pi\) degrees (angle \(\Omega \)) around the peptide helical axis with respect to the C-terminal lobe. The next two steps of anchoring distance shortening and rotation would bring the two lobes of CaM into positions where they clash with each other; hence no examples of 16-1 and 15-1 anchoring are likely to exist. The fourth step of rotation brings the two lobes into an analogous position to where they are in 18-1 anchoring, but at a shorter distance. The shorter distance is easily accommodated by the flexible linker between the lobes, as seen in the 14-1 structures. Performing the same analysis by using parameters \(L \) and \(\Omega \) (see diagram in Fig. 6; also see Ref. 27, which introduced a virtual dihedral angle similar to \(\Omega \)), we find that the \(\Omega \) values are in agreement with the ideal α\(_h\) helical turn/ residue (\(\Omega = 100\Delta n + \varphi \), where \(\Delta n \) is sequence distance between the anchors, and \(\varphi = 15^\circ \) is the phase shift), whereas the \(L \) distance agrees for 14-1 anchored helices (\(L = 1.5\Delta n \)), but the 17-1 and 18-1 anchored helices are progressively more stretched out.

Given that the positioning of the CaM lobes in terms of the peptide anchors is adequately explained by these geometric considerations, the question arises as to the importance of the amphipathic properties of the peptides (28–30). One would...
expect the distribution of hydrophobic residues along the peptide sequences to be conserved relative to the position of the anchors to accommodate the fixed environment of CaM residues. However, there is only one conserved hydrophobic position (position $-3$ from anchor 2) besides the anchor residues (Scheme 1). In addition, there are two positions of conserved hydrophilic (mainly basic) residues (positions $+2$ and $+8$ from anchor 1; see Scheme 1). Overall, this suggests a predominant influence of the two anchors in positioning the CaM lobes in the Phe-Trp type scheme of peptide anchoring.

The same analysis should be applicable to other holoCaM-peptide complexes provided that the peptide anchors are buried in the main hydrophobic pockets of the CaM lobes (pointing toward the $\beta$-sheet contacts of EF hands) and that the peptide is fully helical between the anchors. In supplemental Table S1 we list structural parameters for 18 complexes that fulfill the requirement; these complexes are almost all of the wraparound holoCaM-peptide structures reported in the Protein Data Bank that have peptide anchors separated by at least nine residues. Structures with anchor separation shorter than nine residues were omitted because in these cases a helical peptide is unable to reach into the main hydrophobic pockets of both lobes of holoCaM in the manner described above. A few structures from the Protein Data Bank were excluded because of interference of a third peptide anchor. The regular behavior of $L$ and $\Omega$ as a function of the anchor sequence distance holds for all 18 complexes (diagram in Fig. 6). Because of CaM lobe clashing and linker length restrictions, only $\cos(\Omega) \sim -1$ cases appear to be allowed, limiting the relative position of the second anchor to 10, 14, 17, and 18. This is exactly as expected, assuming that the positions of the CaM lobes are dictated by the relative positions of anchors in the $\alpha_13$-helix of a target peptide,
with some delimitating value of $\Omega$ to avoid lobe clashing. Indeed, all of the excluded positions of the second anchor, 11, 12, 13, 15, and 16, would have $\cos(\Omega) < -0.3$, which corresponds to $\Omega < 100^\circ$, i.e. the lobes would be crowded on the same side of the helix. Lobe clashing does not, however, explain the large preponderance of complexes with the 14-1 anchoring (>70%). This preference is a consequence of the beneficial hydrophobic interactions between the two lobes of holoCaM, which are best realized in the 14-1 anchoring (Fig. 7). By contrast, the new 18-1 anchored structure of the holoCaM-C28 complex shows reduced interlobe interactions of CaM, and the wrapping of the two lobes around the C28 peptide differs significantly from that in comparable (14-1) CaM peptide complexes. In these structures, the two lobes of CaM tightly wrap around the peptide like a clam shell, thereby hiding the CaM-binding domain and shielding it from the solvent. In the 18-1 CaM-C28 complex (and to lesser extent in the 17-1 complex), the two lobes remain separated with few contacts between them, leaving large parts of the C28 CaM-binding domain exposed to solvent (Fig. 7, right column).

### TABLE 2
Comparison of the holoCaM-C28 structure with the structures of related complexes

In the third column, the structure of the backbone heavy atoms of the N-terminal lobe of CaM in its structure with C28 was compared with the structures of the N-terminal lobes of the other CaM-peptide complexes. In the fourth column, the same was done with the C-terminal lobe. In the fifth and sixth columns, the positions of the residues in the anchoring sites are compared for the sets of residues upon alignment of their heavy atoms. The Trp site contains, besides the Trp anchor from the peptide, the following CaM residues: 92, 100, 105, 124, 125, 128, 136, 141, 144, and 145. Analogously, the Phe site contains the CaM residues 19, 27, 32, 52, 55, 63, 68, and 71. NA, not applicable.

| Structures (type: anchors) compared with the NMR structure of holoCaM-C28 2kne (18-1:Trp-1093–Phe-1110) | RMSD |
|---------------------------------------------------------------|------|
| Method (Ref.) | Backbone 10–73 | Backbone 84–145 | Trp anchor site | Phe anchor site |
| 2bcx (17-1:Trp-3620–Phe-3636) | X-ray (31) | 0.76 | 0.72 | 0.57 | 0.94 |
| 2fot (14-1:Trp-1192–Phe-1205) | X-ray (36) | 0.88 | 0.72 | 0.71 | 1.06 |
| 2bbm (14-1:Trp-4–Phe-17) | NMR (21) | 0.86 | 1.08 | 1.00 | 1.27 |
| 1cfl (holoCaM/C20) (None-1:Trp-8) | NMR (7) | NA | 1.24 | 1.27 | NA |

### SCHEME 1
Sequence comparison of peptides bound by CaM via the Phe-Trp anchoring mode. The anchor positions are indicated (Anch.). Conserved hydrophobic and hydrophilic residue positions are boxed.

### FIGURE 6
Positioning of CaM lobes around the helical peptide in the anti-parallel Phe-Trp type anchoring. The dihedral angle $\Omega$ is defined by the C$^\alpha$ atoms of residues lle-27(CaM)-Phe-Trp-Val-136(CaM), and distance $L$ is between the C$^\alpha$ atoms of the Trp and Phe anchors. The diagram on the bottom right shows the dependence of $\Omega$ and $L$ on the relative position of the second anchor ($\Delta n+1$), using data of structures (21, 24, 31, 32, 36–45) in supplemental Table S1 (solid circles denote cases of the anti-parallel Phe-Trp type anchoring). The values expected for an ideal D-helix are represented by the solid line. The “forbidden” positions are marked by ×.

### FIGURE 7
Space filling models of the holoCaM lobes (N-terminal lobe in blue, C-terminal lobe in red, peptide in green) from the four structures representing the four types of lobe anchoring to a helical peptide. The left column structures (without peptide ligand) show the lobe contacts; the right column structures (with ligand) show peptide exposure.
In light of the distance constraints imposed on the anchoring residues discussed above, the case of the peptide from the CBD of rat Ca\(^{2+}\)/CaM-dependent protein kinase kinase is interesting because it has the second anchor at position 16 from the first anchor (31). According to the geometrical model presented above, if the anchors direct CaM wrapping around an \(\alpha\)-helix, this would be a strongly prohibited case (\(\cos(\Omega) = 0.7\)). Remarkably, the structure of the complex of this peptide with holoCaM reveals a partial loss of the helical content of the peptide and an uncharacteristic hairpin looping out of the peptide (31), thus avoiding the restrictions of the model. Moreover, the sense of the peptide binding to CaM is reversed to a parallel mode 1–16, contrary to all other cases of Phe-Trp peptide anchoring (supplemental Table S1). Only one similar structure has been reported (Protein Data Bank entry 1iq5 (32)), which may indicate the exceptionality of this mode of binding.

A complete geometrical description of CaM lobe positioning around a peptide helix requires additional parameters besides \(L\) and \(\Omega\) (supplemental Scheme S1). Most of these parameters are fairly constant through the series of CaM-target peptide complexes considered here.

**Binding Steps Correlate to CaM-C28 and CaM-C20 Structures**—A consensus model of holoCaM binding to PMCA4b (9, 33, 34) involves at least two-step kinetics. In the first step, the C-terminal domain of CaM binds to the N-terminal half of the CBD (involving the anchor Trp-1093) to form a structure analogous to that of holoCaM-C20. In the next step the N-terminal lobe of CaM is engaged and wraps around the CBD to form the final structure. Because the final holoCaM-C-tail complex and the holoCaM-C28 complex have very similar NMR signatures (supplemental Fig. S3), their structures are analogous, which means that atomic resolution structural models of both binding steps are now available. When comparing the two structures, we first note that holoCaM-C28 and holoCaM-C20 can be readily superimposed in the region of the C-terminal lobe and the first 18 residues of the peptides (Fig. 8). Consequently, no substantial structural change appears to be required in the “core” region between the intermediate and the final kinetic binding steps. The N-terminal domain in the holoCaM-C20 ensemble of NMR structures assumes many orientations relative to the core region (7), and the majority of these put the N-terminal lobe of holoCaM far away from the peptide and thus make interaction with the second anchor impossible (Fig. 8, top panel). Only few structures from the ensemble have the N-terminal lobe of holoCaM in proximity with the peptide (Fig. 8, bottom panel). This suggests that the kinetic step of holoCaM N-terminal lobe binding to the C-terminal portion of the peptide is a relatively slow process, as has indeed been observed (33).

During formation of the final wraparound structure of CaM, some subtle adjustments of the peptide binding to the C-terminal lobe appear to happen. NMR chemical shifts of the aromatic

![FIGURE 8. Overlay (stereo view) of representative structure from the holoCaM-C28 ensemble (model 1 in our Protein Data Bank deposition 2kne) and representative (top) or rare (bottom) structures from the holoCaM-C20 ensemble (models 1 and 5 from Protein Data Bank entry 1cff (7)). Structures are aligned in their C-terminal domain (red). The N-terminal domains of holoCaM in the C28 and C20 complex are color-coded dark and light blue, respectively. The peptides C28 and C20 are color-coded green and magenta, respectively.](image-url)
rings of the anchoring Trp-1093 (Trp-8), which are buried deep in the C-terminal lobe of holoCaM, are different in the C20 and C28 complexes. Structural comparison shows that this anchoring site is more different in the C20 and C28 complexes than what is seen between the C28 complex and other complexes analyzed in Table 2. A closer comparison reveals a relative rotation of the Trp side chain rings by ~60 degrees between the two structures. Such rearrangements may be the basis for a third kinetic step invoked in some models of C28 binding to holoCaM (9, 34).

Conclusion—The NMR solution structure of the holoCaM-C28 complex is an excellent surrogate for the holoCaM complex with the whole C-tail from PMCA4b. Our data thus allow the first detailed structural comparison between the initial binding step (represented by the structure of holoCaM-C20) and the final bound complex (holoCaM-C28). A subtle repositioning of the Trp-1093 anchor inside the C-terminal lobe of CaM is indicated, but overall structural comparison supports the role of the CaM-C20 complex as a kinetic intermediate (9, 34) on the path to the final binding mode of holoCaM to the full-length CBD of PMCA4b. It is important to note that although the extended conformation of the previously reported CaM-C20 complex allows a weakening of autoinhibitory interactions of the PMCA and thus (partial) activation of the pump (7), the final, stable complex of CaM and the full-length CaM-binding domain of the PMCA corresponds to the collapsed 18-1 structure reported here. Remarkably, the 18-1 mode of CaM wrapping around the PMCA4b CaM-binding domain leaves a large portion of this domain exposed to solvent and thus potentially available for additional protein interactions. This may be significant for the regulation of the PMCA in its CaM-bound conformation.

The novel binding motif of the holoCaM-C28 complex, in which the helical peptide second anchor is at relative position 18 from the first anchor, extends our knowledge of the modalities of the α₁₆-helix-directed anchoring of holoCaM to target peptides. We found that a model of α₁₆-helix-directed anchoring based on geometrical constraints can accurately predict which helical residue separations between anchors are allowed. Thus, both positions 18 and 17 of the second anchor are permissible, but shorter separations are limited to a four-residue cycle of 14 and 10. Notably, most of the known examples of holoCaM-peptide structures belong to the α₁₆-helix-directed anchoring class.

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