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Structural Consequences of Calmodulin EF Hand Mutations

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Abbreviations: CaM, Calmodulin; iNOS, inducible nitric oxide synthase; CaM-iNOS complex, CaM-iNOS binding domain peptide in complex; HSQC, heteronuclear single-quantum coherence; PDB, Protein Data Bank; apoCaM, Ca^{2+} deplete CaM; CaM_{12}, CaM protein defective in Ca^{2+} binding in the N-lobe EF hands with CaM D20A and D56A mutations; CaM_{34}, CaM protein defective in Ca^{2+} binding in the C-lobe EF hands with CaM D93A and D129A mutations; CaM_{1234}, CaM protein defective in Ca^{2+} binding in all 4 EF hands with CaM D20A, D56A, CaM D93A and D129A mutations; r.m.s.d., root-mean-square distance; APBS, Adaptive Poisson-Boltzmann Solver; BMRB, Biological Magnetic Resonance Bank; SPR, surface plasmon resonance.

ABSTRACT: Calmodulin (CaM) is a cytosolic Ca^{2+}-binding protein that serves as a control element for many enzymes. It consists of two globular domains, each containing two EF hand pairs capable of binding Ca^{2+}, joined by a flexible central linker region. CaM is able to bind and activate its target proteins in the Ca^{2+}-replete and Ca^{2+}-deplete forms. To study the Ca^{2+}-dependent/independent properties of binding and activation of target proteins by CaM, CaM constructs with Ca^{2+} binding disrupting mutations of Asp to Ala at position one of each EF hand have been used. These CaM mutant proteins are deficient in binding Ca^{2+} in either the N-lobe EF hands (CaM_{12}), C-lobe EF hands (CaM_{34}), or all four EF hands (CaM_{1234}). To investigate potential structural changes these mutations may cause we performed detailed NMR studies of CaM_{12}, CaM_{34}, and CaM_{1234} including determining the solution structure of CaM_{1234}. We then investigated if these CaM mutants affected the interaction of CaM with a target protein known to interact with apoCaM by determining the solution structure of CaM_{34} bound to the iNOS CaM binding domain peptide. The structures provide direct structural evidence of changes that are present in these Ca^{2+} deficient CaM mutants and shows these mutations increase the hydrophobic exposed surface and decrease the electronegative surface potential throughout each lobe of CaM. These Ca^{2+} deficient CaM mutants may not be a true representation of apoCaM and may not allow for native-like interactions of apoCaM with its target proteins.

INTRODUCTION

Calmodulin (CaM) is a small cytosolic Ca^{2+}-binding protein able to bind and regulate hundreds of different intracellular proteins.[1] CaM's structure consists of two globular domains, each containing two EF hand pairs capable of binding to Ca^{2+}. These two domains are joined by a flexible central linker region that allows it to adapt its conformation to optimally associate with its intracellular targets.[2] The EF hand consists of a helix-loop-helix motif, consisting of a 12 residue long Ca^{2+} binding loop rich in aspartates and glutamates.[3] In the absence of Ca^{2+} the helix-loop-helix motif of the EF hands are in a "closed" conformation, with their hydrophobic residues packed into their central core and their charged, hydrophilic residues solvent-exposed.[4,5] When a Ca^{2+} ion binds, the helices rearrange into a more "open" conformation, exposing hydrophobic patches in each domain that allow CaM to bind to its target proteins.[3,4,6] CaM is able to bind to its target proteins in the Ca^{2+}-replete and Ca^{2+}-deplete forms.[7–9] There is considerable interest in obtaining a better understanding of the structural basis for CaM's ability to bind and recognize its numerous target proteins.

Mutant CaM proteins are commonly used to investigate the Ca^{2+}-dependent and independent properties of CaM binding and activation of target proteins. These include mutation of the Asp residue to Ala at position 1 to inactivate Ca^{2+} binding in the EF hand.[10–13] These CaM mutants are unable to bind Ca^{2+} in either the N-terminal lobe EF hands (CaM_{12}; CaM D20A and D56A mutations), the C-terminal lobe EF hands (CaM_{34}; CaM D93A and D129A), or all four Ca^{2+}-binding EF hands (CaM_{1234}; mutations at D20A, D56A, D93A and D129A inclusive). Many studies have expressed these Asp to Ala CaM mutants in cells to determine the relative functional contribution of Ca^{2+} binding to each lobe of CaM.[14–34] The overexpression of CaM proteins carrying EF hand mutations in cell culture studies of calcium channels is a commonly

used method for investigating native molecular mechanisms of CaM and the functional contribution of Ca^{2+}.[15-20,30-33] Meaningful interpretation of these investigations should account for differences in the structural integrity of the native apoCaM and the CaM mutant proteins carrying defective Ca^{2+}-binding EF-hands.

The mutation of D93 and D129 to Ala effectively prevents Ca^{2+} binding to EF hands III and IV, however, it has been shown these mutations may cause structural perturbations in the C-domain.[13] This suggests that the Ca^{2+}-inactivating mutations may cause CaM to adopt a non-native apo structure. To investigate these potential structural changes we performed detailed NMR structural studies of CaM_{12}, CaM_{34}, and CaM_{1234} in the absence and presence of Ca^{2+}. The solution structure of CaM_{1234} was determined to examine the effects of these mutations compared to the solution structure of apoCaM. This structure shows that the Asp to Ala mutation cause slight structural and electrostatic surface changes throughout each EF hand.

To further our investigation of these CaM mutants, NMR studies were performed on their association with the CaM binding domain from inducible nitric oxide synthase (iNOS). The iNOS CaM binding domain interacts with the Ca^{2+}-replete and Ca^{2+}-deplete forms of CaM.[35–37] The iNOS enzyme catalyzes the production of nitric oxide (•NO) that acts as a secondary inter- and intracellular messenger involved in many physiological processes.[38] It consists of a dimerized oxygenase domain, flanked by two separated reductase domains, that exist in an equilibrium of conformations alternating between FAD-FMN electron transfer (input state) and FMN–heme electron transfer (output state).[39–41] CaM activates NOS through the precise positioning of the FMN subdomain necessary for the transferring of electrons to the oxygenase domain.[41,42] The iNOS enzyme binds to CaM at basal levels of Ca^{2+} and is transcriptionally regulated *in vivo* by cytokines.[43,44] A previous study of iNOS activity using CaM_{12} and CaM_{34} to

determine if the Ca^{2+}-free N- or C-lobe of CaM was responsible for the Ca^{2+}-independent association of CaM to iNOS showed that iNOS was active for both in the presence of Ca^{2+}, whereas in the presence of EDTA CaM_{34} showed a substantial decrease in iNOS activity.[24] We thus determined the solution structure of CaM_{34} bound to the iNOS CaM binding domain peptide and compared that to the previously determined holoCaM-iNOS complex to provide further insight into this decrease in activity. This structure, along with that of CaM_{1234} showed that the Asp to Ala mutations are associated with an increase in surface hydrophobicity and a decrease in the electronegative surface potential throughout each lobe of CaM. These structures show these mutations may not be a true representation of apoCaM and may not allow for native-like interactions of apoCaM with its target proteins.

EXPERIMENTAL PROCEDURES

Sample Preparation for NMR Investigation. CaM_{12}, CaM_{34} and CaM_{1234} for NMR experiments were expressed in E. coli in 1 L of M9 media (11.03 g/L $Na_2HPO_4 \cdot 7H_2O$, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 3 μM $(NH_4)_6(MO_7)_{24}$, 400 μM H_3BO_3, 30 μM $CoCl_2$, 10 μM $CuSO_4$, 80 μM $MnCl_2 \cdot 4H_2O$, 10 μM $ZnCl_2$, 10 mM $FeSO_4$, 100 μg/mL kanamycin) containing 2 g/L ^{13}C-glucose and 1 g/L $^{15}NH_4Cl$. ^{13}C-^{15}N CaM was purified as previously described.[24] Purity of the mutant CaM protein (148 residues) was confirmed by ESI-MS and was judged to be >95% by SDS-PAGE. The human iNOS peptide (RREIPLKVLVKAVLFACMLMRK, 22 residues corresponding to residues 510-531 from the full length iNOS protein) was synthesized and purchased from CanPeptide Inc. (Montreal, Canada). The iNOS peptide used for SPR was synthesized with biotin attached to the N-terminus to allow for binding to the sensor chip functionalized with an immobilized coating of streptavidin.

The Ca^{2+}-CaM_{12}, Ca^{2+}-CaM_{34} and CaM_{34}-iNOS samples were prepared for NMR experiments via a buffer exchange into NMR solution (100 mM KCl, 10 mM $CaCl_2$, 0.2 mM NaN_3, 90% H_2O/10% ^2H_2O) at pH 6.0 using a YM10 centrifugal filter device (Millipore Corp., Billerica, USA). The apoCaM_{12}, apoCaM_{34} and CaM_{1234} samples were prepared for NMR experiments via a buffer exchange into NMR solution (100 mM KCl, 0.2 mM EDTA, 0.2 mM NaN_3, 90% H_2O/10% ^2H_2O) at pH 6.0 using a YM10 centrifugal filter device (Millipore Corp., Billerica, USA). All NMR samples contained at least 1 mM CaM in a total volume of 500 µL. The samples were transferred into 5 mm NMR sample tubes and stored at 4°C until required for NMR experiments. NMR experiments on the CaM_{34}-iNOS complex were conducted on samples titrated with iNOS peptide to saturation in a 1:1 CaM:peptide ratio. After each addition a ^{1}H-^{15}N heteronuclear single-quantum coherence (HSQC) spectrum was acquired to monitor complex formation.

NMR Spectroscopy and Data Analysis. NMR spectra were recorded at 25°C on Bruker 600 MHz DRX spectrometers equipped with XYZ-gradients triple-resonance probes (Bruker, Billerica, MA, USA). Spectra were analyzed using the program CARA.[45] Specific assignments of the backbone resonances of the CaM_{12}-iNOS and CaM_{34}-iNOS complexes and CaM_{1234} alone were achieved using a combination of three-dimensional triple-resonance experiments, including HNCA and CBCA(CO)NH and the previously obtained amide chemical shifts of Ca^{2+} saturated CaM with the iNOS peptide as reference.[46–49] Side chain resonances for the CaM_{34}-iNOS complex and CaM_{1234} alone were assigned using the TOCSY-type HC(C)H-TOCSY and (H)CCH- TOCSY experiments.[50] Specific assignments of the iNOS peptide in the CaM_{34}-iNOS complex were obtained from ^{15}N-double-filtered NOESY experiments.[51]

Structure Calculation of the CaM_{34}-iNOS Peptide Complex and CaM_{1234} Alone. Distance constraints for CaM_{1234} were obtained from ^{15}N NOESY-HSQC, $^{13}C_{ali}$ NOESY- HSQC and $^{13}C_{aro}$ NOESY- HSQC spectra. Distance constraints for the CaM_{34}-iNOS complex were obtained from ^{15}N NOESY-HSQC and ^{13}C NOESY- HSQC, and ^{15}N- double-filtered NOESY spectra acquired on samples containing ^{13}C-^{15}N-CaM_{34} and unlabeled peptide.[51–53] In addition, dihedral angle restraints were derived from chemical shift analysis with TALOS+. The structure calculations of CaM_{34}-iNOS peptide complex and of CaM_{1234} alone were performed using CNSsolve version 1.2 and initiated with an extended conformation file.[54] The calculation was run through several iterations of a standard simulated annealing protocol to minimize the energies and the final 20 lowest energy structures were selected.

Accession Numbers. The coordinates and NMR parameters have been deposited in the Protein Data Bank (PDB) and the BioMagResBank (BMRB) and have been assigned PDB entry 5TP5, and BMRB accession number 30195 for CaM_{1234} and PDB entry 5TP6, and BMRB 30196 accession number for the CaM_{34}-iNOS complex.

Electrostatics and Hydrophobicity Calculations. Electrostatic potentials of the CaM−iNOS and CaM_{34}-iNOS structures were calculated using the APBS (Adaptive Poisson-Boltzmann Solver) and PDB2PQR software packages.[55–57] The hydrophobicity surface was calculated using the kdHydrophobicity attribute function of UCSF Chimera version 1.5.3 (build 33475)[58] assigning values according to the amino acid hydrophobicity scale of Kyte and Doolittle.[59] The electrostatic potential and hydrophobicity surfaces were viewed in Chimera.

Surface Plasmon Resonance. SPR experiments were performed with an openSPR instrument (Nicoya Lifesciences, Waterloo, Canada) at 25°C with a 100 μL loading loop and a constant flow rate of 50 μL/min. The iNOS peptide with a biotin moiety added to the N-terminus was bound to

a sensor chip functionalized with an immobilized coating of streptavidin. Binding of CaM was tested in either saturating Ca^{2+} buffer (30 mM MOPS, 100 mM KCl, pH 7.2 and 0.1 mM $CaCl_2$) or 17 nM free Ca^{2+} buffer (30 mM MOPS, 100 mM KCl, pH 7.2, and combination of 10 mM EGTA and 10mM CaEGTA to obtain a final 17 nM concentration of free Ca^{2+}). CaM was injected over the bound biotin-iNOS peptide in concentrations from 10 to 300 nM for 2 minutes to allow association. CaM free saturating Ca^{2+} or 17 nM free Ca^{2+} buffer was passed over the sensor for 12 minutes to allow dissociation. Following each CaM injection, 10 mM HCl was injected to completely dissociate the complex and regenerate the unbound iNOS peptide. Analysis of the data was made using Trace Drawer software (Ridgeview Instruments AB) as recommended by the manufacturer. Kinetic parameters were calculated using global analysis, fitting the data to a simple 1:1 model ($A + B \leftrightarrow AB$) for CaM-, CaM_{34}- and CaM_{1234}-interactions with the iNOS peptide at saturated Ca^{2+}.

RESULTS

NMR Structural Studies Of CaM_{12} and CaM_{34} Indicate Possible Structural Perturbations Caused by the Mutations. ^{1}H-^{15}N HSQC experiments were performed to determine if significant structural changes occur due to mutations in the four EF hands of CaM. The complete backbone assignment of CaM_{1234} and CaM_{12} and CaM_{34} and in the absence and presence of Ca^{2+} was completed. These assignments were used to probe potential structural changes caused by the mutations through an ^{1}H-^{15}N HSQC comparison with apo and holoCaM. Comparing the CaM_{1234} spectrum to that of apoCaM[5], chemical shift changes induced by the 4 EF hand mutations appear for the amides throughout all 4 of the Ca^{2+}-binding EF hands (Fig. 1A). The chemical shift differences for CaM_{12} and CaM_{34} were calculated to determine if the chemical shift differences observed for CaM_{1234} are a sum of the individual differences observed

for the mutated EF hands between apoCaM and CaM_{12}, and apoCaM and CaM_{34}. Fig. 1B and C shows the chemical shift differences calculated from the ^{1}H-^{15}N HSQC spectra overlay of apoCaM, $apoCaM_{12}$ and $apoCaM_{34}$ (Supplemental Fig. S1A). Cross-peaks for amides in the C-lobe of $apoCaM_{12}$ overlap with those of apoCaM, however, amides in the N-lobe, specifically the residues in the Ca^{2+} binding loops of the EF hands, do not overlap with those of apoCaM. Conversely, cross-peaks for amides in the N-lobe of $apoCaM_{34}$ overlap with those of apoCaM, however, amides in the C-lobe, specifically the residues in the Ca^{2+} binding loops of the EF hands, do not overlap with those of apoCaM. This data shows that the Asp to Ala mutations not only knock out Ca^{2+} binding to the EF hands but also cause potential structural changes.

Fig. 1D and E show the chemical shift differences calculated from the ^{1}H-^{15}N HSQC spectra overlay of holoCaM, Ca^{2+}-CaM_{12} and Ca^{2+}-CaM_{34} (Supplemental Fig. 1B). As observed for $apoCaM_{12}$, cross-peaks for the majority of amides in the C-lobe of Ca^{2+}-CaM_{12} overlap with those of holoCaM, and amides in the N-lobe, specifically the residues in the Ca^{2+} binding loops of the EF hands, do not overlap. However, unlike $apoCaM_{12}$ there are a few residues in the C-lobe that are calculated to have a chemical shift difference greater than 0.1 ppm.

Also as observed for $apoCaM_{34}$, cross-peaks for amides in the N-lobe of Ca^{2+}-CaM_{34} overlap with those of holoCaM, and amides in the C-lobe, specifically the residues in the Ca^{2+} binding loops of the EF hands, do not. Like Ca^{2+}-CaM_{12}, residues in the opposite lobe appear to be affected by the mutations also. In the Ca^{2+}-CaM_{34} case there are quite a few N-lobe residues, spread throughout the whole domain, that are calculated to have a chemical shift difference greater than 0.1 ppm.This data suggests that the Asp to Ala mutations not only knock out Ca^{2+} binding to the EF hands where the mutations occur, but also cause potential structural changes in the opposite lobe. Some of the residues that experience the greatest changes are the hydrophobic

residues Phe12, Phe16, Leu18, Phe19 and Met36 in EF hand I and the hydrophobic residues, Val55, Ala57, Ile63 and Phe65 in the Ca^{2+} binding loop of EF hand II. These structural changes appear to not only be located to the specific EF hands that contain the mutation but also to residues of the opposite domain, especially in the case of Ca^{2+}-CaM_{34}.

Solution Structure of CaM_{1234}. To probe these potential conformational changes further the structure determination of $apoCaM_{1234}$ was undertaken. The ^{1}H-^{15}N HSQC spectrum of CaM_{1234} exhibits good resolution and well dispersed signals, indicating a uniform and folded protein structure (Supplemental Fig. 2). The three-dimensional solution structure of CaM_{1234} was determined using multidimensional heteronuclear NMR spectroscopy. The structure of the complex is based on a large number of experimental constraints and is well-defined. Structure and input data statistics are summarized in Table 1. The family of 20 lowest energy structures is shown in Fig. 2A and B. Due to the high degree of flexibility of CaM's central linker it is not possible to superimpose both the C and N-lobes at the same time. Superimposing the ensemble of structures with respect to the N-lobe backbone atoms shows a fairly well overlapped N-lobe of CaM_{1234}, with an r.m.s.d. of 0.4 for the backbone atoms and 0.9 Å for heavy atoms. In contrast, superimposing the ensemble with respect to the C-lobe backbone atoms shows a poorer overlap for CaM_{1234}, as shown by an r.m.s.d. of 0.8 for the backbone atoms and 1.3 Å for heavy atoms. This indicates the N-lobe has a more stable structure than the C-lobe, which has previously been reported to have a well-defined hydrophobic core, compared to a C-lobe with a less defined hydrophobic core in the apoCaM structures.[4,5]

Fig. 2C shows the structure consists of 8 helices, and has the characteristic helix-loop-helix conformation for each EF hand, as observed in other apo and Ca^{2+}-replete structures of CaM.[4,5,60] When the ensemble of structures are superimposed with respect to a specific lobe, the linker

region is shown to be very flexible, as evidenced by the opposite lobe being distributed in different conformations relative to the superimposed lobe. This is also supported by the lack of long range NOEs observed for the linker residues or between the two lobes.

CaM_{1234} Structure Comparison to ApoCaM. The CaM_{1234} structure was compared to the previously determined solution structure of apoCaM (PDB entry 1CFC)[5] to determine any structural changes incurred by the Asp to Ala mutations in position 1 of the four EF hands. Due to the highly flexible linker region described above, the two lobes of CaM of the two structures were compared separately (Fig. 3). When the two structures were superimposed with respect to CaM's N-lobe backbone atoms (residues 4-70) a r.m.s.d. value of 2.9 Å was found. Fig. 3A and B shows the superposition of the N-lobe of CaM_{1234} and apoCaM. Helix A and the loop region connecting the two EF hands overlay quite well in both structures, whereas helix B is shifted down compared to apoCaM and the Ca^{2+}-binding loop of EF hand I is tilted away from the Ca^{2+}-binding loop of EF hand II.

When the two structures are superimposed with respect to the C-lobe backbone atoms (residues 84-148) a r.m.s.d. value of 2.6 Å was found. Fig. 3C and D shows the superposition of the C-lobe of CaM_{1234} and apoCaM, which displays more subtle changes and less drastic conformational changes compared to the N-lobe. The largest differences seen are a bulge of the Ca^{2+}-binding loop of EF hand III at the Asp93 to Ala mutation site and the tilting inwards of helix E.

Effect of EF Hand Mutations on the Electrostatic and Hydrophobicity Surface Maps. The electrostatic surface potential of apoCaM and CaM_{1234} were calculated using the APBS (Adaptive Poisson-Boltzmann Solver) and PDB2PQR software packages and mapped onto the surfaces of the apoCaM and CaM_{1234} solution structures (Fig. 4A, B and D, E, respectively). The

negative surface charge observed throughout apoCaM has been drastically reduced throughout all 4 EF hands of the CaM_{1234} structure. The hydrophobicity surface was also calculated, using Chimera, and mapped onto the surfaces of apoCaM and CaM_{1234} (Fig. 4C and F, respectively). The mutations have reduced the hydrophilic surface area and increased the hydrophobic surface area throughout the two lobes of the CaM_{1234} structure.

NMR Structure of CaM_{34} and the iNOS CaM Binding Domain Peptide Complex. -We next determined the solution structure of CaM_{34} bound to a peptide of a target protein, iNOS, to investigate if these CaM mutants affected the interaction of CaM with target proteins known to interact with apoCaM. Overall, the ^{1}H- ^{15}N HSQC spectrum of CaM_{34} in complex with the peptide of the iNOS CaM-binding domain exhibits good resolution and well dispersed signals, indicating a uniform and folded protein structure (Supplemental Fig. S3A). Upon comparison with the ^{1}H- ^{15}N HSQC spectrum of the holoCaM complex chemical shift changes induced by the C-lobe EF hand mutations appear predominately for the amides coordinating the Ca^{2+} ion in EF hands III and IV, with the greatest differences occurring for the amides in the center of the Ca^{2+}-binding loop (Supplemental Fig. S3B). The majority of the amide resonances of the N-lobe show little chemical shift differences, suggesting both complexes have a similar structure of the N-lobe bound to iNOS, however, a few meaningful differences are observed.

The three-dimensional solution structure of CaM_{34}-iNOS complex was determined using multidimensional heteronuclear NMR spectroscopy. The structure of the complex is based on a large number of experimental constraints and is well-defined. Residues 1− 12 at the N-terminus of the iNOS peptide (corresponding to residues 503-514 of full length iNOS) show a lack of structure because they could not be unambiguously assigned and were omitted from the structure

calculation. The root-mean-square distance (r.m.s.d.) for ordered residues is 1.0 Å for the backbone atoms and 1.4 Å for all non-hydrogen atoms (Table 2).

The family of 20 lowest energy structures is shown in Fig. 5A. This ensemble of structures shows a more closely overlapped N-lobe of CaM_{34} compared to a C-lobe that displays more fluctuation in the ensemble of structures. This can be further shown by looking at the r.m.s.d. values for each individual lobe of CaM_{34} in complex with the iNOS peptide. The r.m.s.d. for the C-lobe residues is 1.0 Å for the backbone atoms and 1.7 Å for all non-hydrogen atoms, whereas it is 0.7 Å for the backbone atoms and 1.2 Å for all non-hydrogen atoms of the N-lobe. The CaM_{34}-iNOS complex has a Ca^{2+}-replete N-lobe and a Ca^{2+}-deplete C-lobe bound to the iNOS peptide as shown in Fig. 5B. This structure shows CaM is still able to bind to iNOS with both lobes, even when the C-lobe of CaM is Ca^{2+}-deplete due to the Asp to Ala mutations.

CaM_{34}-iNOS Structure Comparison to the holoCaM-iNOS Complex and ApoCaM. When the CaM_{34}-iNOS complex structure is compared to the previously determined solution structure of the holoCaM−iNOS complex (PDB entry 2LL6)[46], the N-lobes of CaM and peptide orientation are quite similar, however the Ca^{2+} binding loops of EF hands III and IV of the C-lobe of CaM are structurally different (Fig. 6A-D). When the two structures are aligned with respect to CaM_{34}-iNOS's backbone atoms a r.m.s.d. value of 4.2 Å for the backbone atoms of CaM-iNOS was found. When the two structures are aligned with respect to CaM's N-lobe backbone atoms a r.m.s.d. value of 2.5 Å was found, whereas, an r.m.s.d. value of 3.2 with respect to the C-lobe backbone atoms was found. The N-lobes of CaM and the iNOS peptide of each structure superimpose quite well on each other, whereas the Ca^{2+} binding loops of EF hands III and IV of the C-lobe of CaM do not. Even though the C-lobe of CaM_{34} is Ca^{2+}-deplete, the four α-helices of the two EF hand loops still bind to the iNOS peptide in a similar fashion as in

the holoCaM-iNOS complex. This is evident from the solution structure and also from the NOESY spectra. The inter-residue NOEs observed for the residues of the α-helices of CaM_{34}'s C-lobe are very similar to those observed for the same residues in the holoCaM-iNOS complex. The Ca^{2+} binding loops of EF hands III and IV are more compact in the holoCaM-iNOS complex compared to the CaM_{34}-iNOS complex and the loop region connecting EF hands III and IV is closer to the peptide in the CaM_{34}-iNOS complex. The loop region connecting EF hands I and II and helix B are also shifted closer to the iNOS peptide.

When the CaM_{34}-iNOS complex structure is compared to the previously determined apoCaM structure (PDB entry 1CFC)[5], there is structural similarity of the Ca^{2+} binding loop of EF hand III of the C-lobes of CaM (Fig. 6E and F). When the two structures are aligned with respect to CaM's EF hand III Ca^{2+}-binding loop backbone atoms (residues 93-104) a r.m.s.d. value of 1.1 Å was found. When the two structures are aligned with respect to CaM's C-lobe backbone atoms (residues 93-140) a r.m.s.d. value of 3.5 Å was found. The structure and r.m.s.d. values suggest the EF hands of the C-lobe adopt a similar Ca^{2+}-deplete conformation for the Ca^{2+}-binding loop, however the overall conformation of the helix-loop-helix motif is similar to the "open" conformation observed in the Ca^{2+}-replete form (Fig. 6F).

Effect of EF Hands 3 and 4 Mutations on the Electrostatic and Hydrophobicity Surface Maps of the CaM_{34}-iNOS Complex. The electrostatic surface potentials of holoCaM-iNOS and CaM_{34}-iNOS were calculated and mapped onto their respective surfaces (Fig. 7A and B). As was observed for CaM_{1234}, the EF hand III and IV mutations cause the C-lobe of CaM_{34} to have a less electronegative surface than that of holoCaM-iNOS. Overall the electrostatic surface of the N-lobe for both the holoCaM-iNOS and the CaM_{34}-iNOS structures are quite similar (Supplemental Fig. 4). The hydrophobicity surface was mapped onto the surfaces of holoCaM-iNOS and

CaM_{34}-iNOS (Fig. 7C and D, respectively) and a slight increase of hydrophobic surface area can be observed near the mutation sites in the CaM_{34}-iNOS structure.

Surface Plasmon Resonance of CaM, CaM_{1234} and CaM_{34} with iNOS. The interaction of the iNOS peptide with CaM, CaM_{1234} and CaM_{34} was further analyzed using SPR. SPR analysis allows us to determine the binding kinetics of a protein to a peptide with the advantage of using a label-free system. The kinetics of this binding interaction, including the binding affinity, association and dissociation rates, was determined by the injection of different concentrations of CaM over a surface with an immobilized iNOS peptide.

Sensorgrams at various CaM concentrations (10 to 300 nM) were obtained at a saturating Ca^{2+} concentration of 1 mM with the iNOS peptide (Supplementary Fig. S5). The sensorgrams consisted of three phases: an association phase, injection of buffer with CaM; a dissociation phase, flushing of the flow cell with CaM-free buffer; and a regeneration phase, injection of 10 mM HCl for complete dissociation of the complex. Sensorgrams were analyzed by a simple 1:1 (A + B ↔ AB, where A is CaM and B is the immobilized iNOS peptide) fitting model using the Tracedrawer software (Ridgeview Instruments AB) (Table 3).

We determined the association rate, k_a, to be 27.5 (± 0.01) x 10^4 $M^{-1}s^{-1}$ and the dissociation rate, k_d, to be 3.9 (± 0.02) x 10^{-4} s^{-1}, giving a dissociation constant, K_D, of 1.4 (± 0.01) x 10^{-9} M (Table 3) for CaM at saturating Ca^{2+}, which is in good agreement with previously reported values that range from <0.1–3.3 x 10^{-9} M.[61–65] To mimic the basal Ca^{2+} normally found in the cell SPR experiments were performed at 17 nM free Ca^{2+} concentration since previous structural studies have shown little change in overall CaM structure when bound to iNOS under these conditions compared to saturated Ca^{2+} concentrations.[66] Sensorgrams were then obtained at the 17 nM free Ca^{2+} concentration to evaluate the binding kinetics of CaM with the iNOS peptide.

This yielded a k_a of 20.5 (± 0.02) x 10^4 $M^{-1}s^{-1}$, k_d of 14.5 (± 0.2) x 10^{-4} s^{-1} and an apparent K_D of 7.1 (± 1.0) x 10^{-9} M. To evaluate the binding kinetics of the CaM mutants investigated by NMR to the iNOS peptide we performed SPR experiments at saturating Ca^{2+}. The binding of CaM_{1234} to the immobilized iNOS peptide resulted in a K_D of 2.9 (± 0.1) x 10^{-9} M with both slower on and off rates, whereas CaM_{34} binding to iNOS had faster on and off rates with a K_D of 1.9 (± 0.1) x 10^{-9} M.

DISCUSSION

To study the Ca^{2+}-dependent/independent properties of binding and activation of target proteins by CaM, numerous studies use a series of CaM mutants that inhibit Ca^{2+} binding to the EF hands. These include mutation of Asp to Ala at position 1 of each EF hand in the N-lobe or C-lobe or both lobes.[10–13] Many studies have expressed CaM_{12}, CaM_{34} or CaM_{1234} in cells to determine if the Ca^{2+}-independent association of CaM to its target proteins is due to the Ca^{2+}-free N- or C-lobe of CaM.[14–33] These point mutations have been reported to result in structural changes in the protein in addition to knocking out its ability to bind Ca^{2+}. Due to the widespread use of these CaM EF mutants, further investigation of their structures in solution was warranted.

The CaM_{1234} structure was compared to the previously determined solution structure of apoCaM[5] to determine any structural changes incurred by the Asp to Ala mutations in position 1 of the four EF hands. The biggest structural change observed is in the Ca^{2+}-binding loop of EF hand I. In the apoCaM structure the side chain of Asp20 points into the Ca^{2+} binding loop and is involved in hydrogen bonds that stabilize the loop. The conversion of Asp to Ala in the CaM_{1234} structure causes the Ca^{2+} binding loop to have a less defined structure by eliminating these hydrogen bonds and disrupting the antiparallel β-sheets connecting EF hands I and II. This also causes the loop and helix B to tilt away from the loop of EF hand II.

The Ca^{2+}-binding loop of EF hand II also displays structural changes, however these aren't as large as those observed for EF hand I. In the apoCaM structure the side chain of Asp56 is exposed to the solvent, thus doesn't have as large of a role in stabilizing the loop structure and could explain the lower degree of structural change. In the CaM_{1234} structure the substituted Ala56 side chain points into the loop, disrupting the α-helix that Asp56 adopted, unraveling the loop slightly and tilting helix C towards helix D. The linker region displays similar α-helical secondary structure for helices D and E, with a hinge region at residue 80 for both structures.

As in the case of EF hand I, the side chain of Asp93 of EF hand III points into the loop, however, the mutation to Ala doesn't cause as large of a structure perturbation as Asp20 to Ala. The packing of the Ala side chain into the loop toward the other hydrophobic side chain groups causes the loop to bulge compared to apoCaM. The Asp129 to Ala mutation causes more subtle changes in the Ca^{2+}-binding loop of EF hand IV and helix G. The loop region connecting the two EF hands has a slightly different conformation due to helix G being tilted inward. Overall the N-lobe mutations cause more overall conformational changes compared to the C-lobe, as evidenced by the higher r.m.s.d. value for the lobe and the aforementioned differences. These structural changes also correlate well with the chemical shift differences observed between the structures, which shows the N-lobe of CaM_{1234} has larger differences in the Ca^{2+}-binding loops, but fewer differences in the rest of the N-lobe, whereas the C-lobe shows lower chemical shift difference values in the Ca^{2+}-binding loop but a larger amount of subtle differences throughout the whole lobe.

These structural changes also cause electrostatic changes on the surface of CaM, which may affect how CaM_{1234} interacts with CaM's target enzymes outside of their CaM binding domains. It has previously been shown that CaM lobe surface interactions with regions of its target

enzymes outside of their CaM binding domains are integral for full enzyme activity.[67–71] Even though CaM_{1234} mimics the Ca^{2+} free nature of apoCaM, the changes in electrostatic surface potential may not allow for full native-like apoCaM interactions with CaM's target proteins.

The solution structure of CaM_{34} bound to iNOS was determined to investigate if these CaM mutants affected the interaction of CaM with target proteins known to interact with apoCaM. The complex of CaM_{34} with iNOS was chosen for several reasons. First, binding of CaM to iNOS is Ca^{2+}-independent, so a complex will be formed even with the Ca^{2+}-deplete C-lobe. Second, it would allow us to determine if the EF hand III and IV mutations cause structural changes in the N-lobe as observed from the chemical shift differences between holoCaM and Ca^{2+}-CaM_{34} in Fig. 1E. Finally, because in previous iNOS activation studies with the mutant CaMs, CaM_{34} was the only one to have differences in activation of iNOS in the presence and absence of Ca^{2+}.[24]

The CaM_{34}-iNOS structure was compared to the previously determined solution structures of holoCaM-iNOS apoCaM. The N-lobes of CaM_{34} and the iNOS peptides are quite similar to the holoCaM-iNOS structure, whereas the Ca^{2+} binding loops of EF hands III and IV display differences between the structures. The mutation of Asp to Ala removes the oxygen ligand necessary to coordinate a Ca^{2+} ion and causes the Ca^{2+} binding loops of EF hands III and IV to adopt a less compact conformation in the CaM_{34}-iNOS structure. This causes local structural changes, such as the loop region connecting EF hands III and IV to move closer to the iNOS peptide, and long range structural conformation changes, observed in the loop region connecting EF hands I and II and helix B. The loop regions connecting EF hands I and II and connecting EF hands III and IV contain multiple hydrophobic residues that pack close together and interact with the hydrophobic residues of the iNOS peptide. This may account for the amide chemical shift

changes in the N-domain of Ca^{2+}-saturated CaM_{34} that were previously observed.[13] The EF hands of the C-lobe of CaM_{34} have a Ca^{2+}-deplete conformation for the Ca^{2+}-binding loop similar to apoCaM; however the interaction with the highly hydrophobic iNOS peptide causes the overall conformation of the helix-loop-helix motifs to be similar to the "open" conformation observed in the Ca^{2+}-replete form. The mutations also cause a decrease in the electronegative surface potential of CaM's C-lobe, which may cause nonnative-like apoCaM interactions with other regions of the iNOS enzyme. These structures can be used to explain previous iNOS activity studies using CaM and the Ca^{2+} deficient CaM mutants.

One of these studies found that iNOS was active for both CaM_{34} and CaM_{12}, with CaM_{34} rates similar to holoCaM and CaM_{12} rates similar to apoCaM, in the presence of Ca^{2+}, whereas CaM_{1234} produced significantly reduced rates. In the presence of EDTA a substantial decrease in iNOS activity was found for wild type CaM and CaM_{34}, whereas no substantial decrease in iNOS activity was found for CaM_{12} or $CaM_{1234.}$[24] For full activation of iNOS by CaM it has been shown that not only is binding of CaM to iNOS' CaM binding domain required, but interaction of CaM with both the oxygenase and reductase domains of iNOS is also required to stabilize the iNOS output state.[69,70,62] The interaction of CaM with iNOS is largely electrostatic; with the positive residues at the heme and FMN domain interfaces interacting with the negative residues of CaM's N- and C-lobes.[68,72,73] Molecular dynamics, X-ray crystallography and electron cryo-microscopy studies have been used to determine key interactions between CaM residues and residues of the FMN and both heme domains of the homodimer in the input and output states (supplemental Table S1).[68,71–73] Some potential important connections required for full activity of iNOS are: Glu47 of CaM with Arg536 of the FMN domain; Asp122 of CaM with Arg83 and Arg86 of the inter monomer heme domain; and Arg106 of CaM with Glu285 of the intra

monomer heme domain. These studies also showed that CaM undergoes conformational changes, along with the FMN and heme domains, from the input to the output state. During the movement of the FMN domain to the output state the EF hand loops III and IV move by about 2 Å and 5 Å, respectively, due to the shifting of the CaM binding domain, while the N-lobe has a much larger conformational change, due to its interaction with the FMN domain.[73] All of these interactions, in addition to others observed (supplemental Table S1), are kept fully intact in the CaM_{34}-iNOS structure. The CaM_{34} mutation causes the conformation of the C-lobe of CaM to resemble that of CaM interacting with the heme domain in the output state, specifically Arg106 of EF hand III moves about 3 Å and residues Glu122 and Glu127 of EF hand IV move about 6 Å and 5 Å, respectively (Fig. 8). Meanwhile, helix E of the C-lobe of CaM_{34}, which is found to interact with the FMN domain, does not move relative to holoCaM. The CaM_{34} mutation may cause the C-lobe of CaM to be in a conformation that has interactions which stabilizes the output state conformation of the heme domains, while still allowing the unaffected N-lobe of CaM to have the conformational movement with the FMN domain that is necessary to facilitate the inter domain electron transfer. The N-lobe of the CaM_{34}-iNOS structure is bound to iNOS in the same conformation as holoCaM (Figs. 6 and 8), which explains the full activity of iNOS in the presence of Ca^{2+}. Also the N-lobe of CaM alone has previously been shown to activate the iNOS enzyme in the presence of Ca^{2+}, thus its tight association with iNOS is enough to fully activate iNOS.[68,74,75]

The reduced iNOS activity observed for CaM_{34} in the presence of EDTA could be caused by the rearrangement of EF hands I and II due to the removal of Ca^{2+} from the N-lobe, which would affect the residues of helix C (Fig. 8) that interact with the FMN domain. This conformational change, coupled with the affected conformation of the loop region between EF hands I and II due

to the CaM_{34} mutation, may not allow for the necessary interactions of the N-lobe of CaM to the FMN domain of iNOS required for efficient electron transfer, although CaM is still bound to the CaM binding domain of the enzyme due to strong hydrophobic interactions. This may prevent the FMN conformational change required for efficient electron transfer to the heme domain or prevent CaM from stabilizing the FMN to heme electron transfer in the "output" state.[41,71,72,76]

The structural rationale for the lower activity observed for CaM_{1234} with iNOS can be explained by comparing the structure of apoCaM to CaM_{1234}. Although CaM_{1234} is still able to bind to the highly hydrophobic CaM-binding domain of iNOS, the structural perturbations and electrostatic surface potential changes induced by the EF hand mutations may affect how CaM_{1234} interacts with the rest of iNOS, specifically CaM's N-lobe interaction with the FMN domain. The less hydrophilic and less negative electrostatic surface of CaM_{1234} would affect its interaction with the positive surface residues of iNOS at the CaM-FMN interface, which have previously been shown to be necessary for full activation of iNOS.[69,72,73] This would prevent CaM's stabilizing interaction with the FMN and heme domains in the output state of holoCaM with iNOS. The lower activity of iNOS with CaM_{12} in the presence and absence of Ca^{2+} may also be due to the structural and electrostatic changes in the CaM N-lobe as discussed above.

The K_D of CaM binding to the iNOS peptide is in good agreement with previously reported values that range from <0.1–3.3 x 10^{-9} M.[61–65] Previous NMR studies have suggested that at the 17 nM free Ca^{2+} concentration iNOS increases CaM's affinity for Ca^{2+} and has a Ca^{2+}-replete CaM structure.[66] The binding kinetics at 17 nM free Ca^{2+} concentration are slower than at saturating Ca^{2+} concentrations due to CaM having to bind to Ca^{2+} and the iNOS peptide, whereas at the saturating Ca^{2+} concentration CaM is already Ca^{2+}-replete and in the correct conformation for binding to iNOS. The slower on and off rates observed for CaM_{1234} binding to iNOS compared to CaM at both 17 nM free Ca^{2+} and saturated Ca^{2+}

concentration could be explained by the structural perturbations introduced in the N-lobe EF Hands that prevent CaM from adopting the correct Ca^{2+}-replete conformation necessary for optimum binding to iNOS. The fully intact N-lobe of CaM_{34} would explain why it has a higher on rate when binding to the iNOS peptide. This is consistent with previous work that has shown only the N-lobe of CaM is necessary for its tight association with iNOS.[68,74,75]

In summary, mutations in the EF hands of CaM to disable Ca^{2+}-binding also cause slight structural perturbations, shown in this study by the use of NMR spectroscopy. The structure determination of CaM_{1234} revealed that the mutation of Asp to Ala causes the EF hand loops to adopt perturbed conformations when compared to apoCaM, with the greatest effect seen in EF Hand I. The structure also displayed a less stable C-lobe compared to N-lobe as previously observed for apoCaM. To investigate if these mutations also perturb the structure of CaM bound to a target peptide the structure of CaM_{34} bound to the iNOS peptide was determined. The mutation of Asp to Ala causes the Ca^{2+} binding loops in the C-lobe EF hands to adopt a conformation resembling apoCaM, which causes local structural changes, as shown in the loop region connecting EF hands III and IV, and long range structural conformation changes, as shown in the loop region connecting EF hands I and II and helix B. These changes may cause CaM to adopt conformations not truly representative of apoCaM. This study provides structural evidence of changes that are present in CaM mutants with mutations at Asp in position 1 of the EF hand. These mutations also increase the hydrophobic exposed surface and decrease the electronegative surface potential throughout each lobe of CaM. To investigate whether the small structural differences between apoCaM and CaM_{1234} result in appreciable changes in biding kinetics with the iNOS peptide we performed SPR experiments on the different CaM proteins. Both the on and off rates for CaM_{1234} binding to the iNOS peptide were significantly slower than to CaM at both 17 nM free

Ca^{2+} and saturated Ca^{2+} concentration. The differences in binding kinetics with CaM at basal Ca^{2+} levels are significant as they provide further reason to carefully scrutinize experiments that use CaM_{1234} to represent the Ca^{2+}-free form of the protein. These Ca^{2+} deficient CaM mutants may not be a true representation of apoCaM and may not allow for native-like interactions of apoCaM with its target proteins.

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SUPPORTING INFORMATION

Superposition of ^{1}H-^{15}N HSQC spectra of apo and Ca^{2+}-saturated wild type CaM, CaM_{12} and CaM_{34}, superposition of ^{1}H-^{15}N HSQC spectra of CaM_{1234} and apoCaM, superposition of ^{1}H-^{15}N HSQC spectra of CaM_{34}-iNOS and holoCaM-iNOS, electrostatic surface representations of CaM-iNOS and CaM_{34}-iNOS, SPR sensorgrams of the binding of CaM, CaM_{1234} CaM_{34} onto immobilized iNOS peptide, residue-residue interaction of CaM with the iNOS heme and FMN domains.

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Table 1: Statistics for the structural ensemble of CaM_{1234}.

	CaM_{1234}			
	NMR-derived distance and dihedral angle restraints			
NOE constraints	short-range (|i − j| < 1)	sequential	medium-range (1 < |i − j| < 4)	long-range (|i − j| > 4)
	968	325	205	265
Long Range Intra EF NOEs[a]	EF I	EF II	EF III	EF IV
	45	47	35	31
Total		1763		
Dihedral angles from TALOS+		240		
Total number of restraints		2003		
	Structure statistics for the 20 lowest energy structures			
Mean deviation from ideal covalent geometry				
Bond lengths (Å)		0.009		
Bond angles (deg.)		0.9		
Average pairwise RMSD (Å) for all heavy atoms of the 20 lowest energy structures	All Residues	Ordered Residues[b]	C-lobe[c]	N-lobe[d]
Backbone Atoms	6.3	5.5	0.8	0.4
Heavy Atoms	6.6	6.0	1.3	0.9
Ramachandran statistics (%)				
Residues in most favored region		87.4		
Residues in additional allowed regions		11.8		
Residues in generously allowed region		0.0		
Residues in disallowed region		0.7		

[a] Long range NOEs observed between residues in the same EF Hand

[b] Ordered residue ranges: 4A-20A,24A-57A,61A-79A,81A-92A,101A-112A,117A-129A,132A-147A

[c] C-lobe residues: 81A-148A

[d] N-lobe residues: 4A-74A

Table 2: Statistics for the structural ensemble of the CaM_{34}-iNOS peptide complex.

		CaM_{34}-iNOS Complex		
		NMR-derived distance and dihedral angle restraints		
	CaM_{34}	iNOS peptide	CaM_{34}-iNOS complex	
NOE constraints				
short-range ($\|i-j\| < 1$)	821	125	N/A	
sequential	497	52	N/A	
medium-range ($1 < \|i-j\| < 4$)	240	33	N/A	
long-range ($\|i-j\| > 4$)	160	4	82	
Total	1718	214	82	
Dihedral angles from TALOS+	262	N/A	N/A	
Total number of restraints		2276		
		Structure statistics for the 20 lowest energy structures		
Mean deviation from ideal covalent geometry				
Bond lengths (Å)		0.010		
Bond angles (deg.)		1.2		
Average pairwise RMSD (Å) for all heavy atoms of the 20 lowest energy structures	All Residues	Ordered Residues[a]	C-lobe[b]	N-lobe[c]
Backbone Atoms	1.3	1.0	1.0	0.7
Heavy Atoms	1.7	1.4	1.6	1.2
Ramachandran statistics (%)				
Residues in most favored region		84.4		
Residues in additional allowed regions		14.4		
Residues in generously allowed region		0.5		
Residues in disallowed region		0.7		

[a] Ordered residue ranges: 5A-42A, 44A-92A, 100A-147A, 517B-528B
[b] C-lobe residues: 81A-148A
[c] N-lobe residues: 4A-74A

Table 3. k_{on} and k_{off} rate constants of iNOS target peptide to CaM in the presence of 17 nM free Ca^{2+} and saturating (1mM) Ca^{2+}.

Saturating Ca^{2+}	**k_{on} (10^4 $M^{-1}s^{-1}$)[a]**	**k_{off} (10^{-4} s^{-1})[a]**	**K_D (10^{-9} M)[a]**
CaM-iNOS	27.5 ± 0.01	3.9 ± 0.02	1.4 ± 0.01
CaM_{1234}-iNOS	6.4 ± 0.1	1.9 ± 0.03	2.9 ± 0.1
CaM_{34}-iNOS	44.2 ± 0.01	8.5 ± 0.04	1.9 ± 0.01
17 nM free Ca^{2+}	**k_{on} (10^4 $M^{-1}s^{-1}$)[a]**	**k_{off} (10^{-4} s^{-1})[a]**	**K_D (10^{-9} M)[a]**
CaM-iNOS	20.5 ± 0.04	14.5 ± 0.2	7.1 ± 1.0

[a] Data was fit in TraceDrawer using a 1:1 binding interaction model (A+B ↔ AB) with errors from TraceDrawer.

FIGURE CAPTIONS

FIGURE 1. Chemical shift differences for the amide chemical shifts between (A) CaM_{1234} and apoCaM (B) apoCaM and $apoCaM_{12}$, (C) apoCaM and $apoCaM_{34}$, (D) holoCaM and Ca^{2+}-CaM_{12}, and (E) holoCaM and Ca^{2+}-CaM_{34} are shown. The insets in D and E show the chemical shift differences of the C-lobe for holoCaM and Ca^{2+}-CaM_{12} and N-lobe for holoCaM and Ca^{2+}-CaM_{34}, respectively. The greatest differences are localized to Ca^{2+} binding loops where each mutation is present. In the presence of Ca^{2+} some chemical shift differences occur for the lobe opposite the mutation sites. The contribution of ^{1}HN and ^{15}N chemical shift changes for each residue was calculated as $\Delta\delta = \sqrt{[(\Delta\delta^{1}HN)^2 + (\Delta\delta^{15}N/5)^2]}$, where $\Delta\delta^{1}$HN and $\Delta\delta^{15}$N are the differences in ^{1}HN and ^{15}N chemical shifts between the indicated protein.

FIGURE 2. Solution structure of CaM_{1234}. The superposition of the ensemble of the 20 lowest-energy calculated solution structures of CaM_{1234}.(A) The N-terminal domain is superimposed against the energy-minimized average structure. (B) The C-terminal domain is superimposed against the energy-minimized average structure. (C) Cartoon ribbon view of the energy-minimized average solution structure of the CaM_{1234}. Residues 1−40 of CaM (EF hand I) are colored red, residues 41−79 (EF hand II) purple, residues 80−114 (EF hand III) green, and residues 115−148 (EF hand IV) blue.

FIGURE 3. Comparison of the solution structure of the CaM_{1234} with the solution structure of apoCaM. The solution structures of CaM_{1234} (dark colors) and apoCaM (light colors, Structure from PDB 1CFD (5)) are overlaid. For clarity only the N-lobes of CaM_{1234} and apoCaM were superimposed in A and B and the C-lobes superimposed in C and D. The side chains of Asp in apoCaM and Ala in CaM_{1234} are shown and labeled as D20A, D56A, D93A and D129A. The

superimposed structures are viewed from EF Hand I to IV in panels A to D. The color scheme is the same as Fig. 2.

FIGURE 4. Electrostatic and hydrophobicity surface representations of apoCaM and CaM_{1234}. The APBS-calculated electrostatic potential maps projected on the surface of (A, B) apoCaM (Solution structure from PDB 1CFD (5)) and (D, E) the CaM_{1234} solution structure. The hydrophobicity surface calculated in chimera, using the Kyte-Doolittle scale, projected onto (C) apoCaM and (F) the CaM_{1234} solution structures. The electrostatic potential maps are colored with a chimera color key ranging from red (acidic, −10) to blue (basic, 10). The hydrophobicity surface is colored from blue (4.5) for the most hydrophilic, to white, to orange red (-4.5) for the most hydrophobic. The mutation sites D20A, D56A, D93A and D129A are labeled and indicated by *.

FIGURE 5. Solution structure of the CaM_{34}-iNOS complex. (A) Superposition of the ensemble of the 20 lowest-energy calculated NMR solution structures of CaM_{34} bound to the iNOS peptide. Backbone atom traces of CaM are colored dark blue, and the iNOS peptide colored light blue. (B) Cartoon ribbon view of the average solution structure of the CaM_{34}-iNOS complex. CaM has the same color scheme as Fig. 2. The peptide is colored lighter blue.

FIGURE 6. (A-D) Comparison of the solution structures of the CaM_{34}-iNOS peptide complex and the holoCaM-iNOS peptide complex. The solution structures of the CaM_{34}-iNOS peptide (dark colors) and holoCaM-iNOS (light colors) are aligned by superimposition of the backbone atoms of the N-lobes of CaM and the iNOS peptides viewed along the bound peptide from its C-terminus (C′) to its N-terminus (N′) in A and subsequently rotated 90° around the vertical axis in B. The superposition is viewed along the bound peptide from its N-terminus (N′) to its C-terminus (C′) in C and subsequently rotated 90° around the vertical axis in D. Calcium ions were

omitted for clarity. (E, F) Comparison of the C-terminal residues of the solution structures of the CaM_{34}-iNOS peptide complex and apoCaM. The solution structures of the CaM_{34}-iNOS peptide (dark colors) and apoCaM (light colors) are aligned by superimposition of the backbone atoms of the C-lobes of CaM. The superimposed structures are viewed along the EF hand III and IV interface in E and viewed along the side of EF Hand IV in F. The N-lobes and the iNOS peptide have been removed for clarity. The color scheme is the same as Fig. 2.

FIGURE 7. Electrostatic and hydrophobicity surface representations of CaM-iNOS and CaM_{34}-iNOS. The APBS-calculated electrostatic potential maps are projected on the surface of the C-lobes of the solution structure of the CaM−iNOS peptide complex (A) and solution structure of the CaM_{34}-iNOS peptide complex (B). The hydrophobicity surface calculated in chimera, using the Kyte-Doolittle scale, projected onto the surface of the C-lobes of the solution structure of the CaM-iNOS peptide complex (C) and solution structure of the CaM_{34}-iNOS peptide complex (D). The electrostatic potential maps and hydrophobicity surfaces are colored as in Fig. 4.

FIGURE 8. The solution structure of the CaM_{34}-iNOS peptide (dark colors) and the crystal structure of the iNOS oxy-FMN-holoCaM complex (CaM in light colors, iNOS CaM binding and FMN domains in yellow) are aligned by superimposition of the backbone atoms of CaM and the iNOS CaM binding domain. The side chains of key residues of CaM that interact with iNOS' heme domains and the distance of their shifts are labeled. The color scheme is the same as Fig. 2. Calcium ions are shown as orange spheres and labeled.