Recent studies have shown that substitution of Ala for one or more Phe residues in calmodulin (CaM) imparts a temperature-sensitive phenotype to yeast (Ohyaa, Y., and Botstein, D. (1994) Science 263, 963–966). The Phe residue immediately preceding the first Ca$^{2+}$ ligand in site III of CaM (Phe-92) was found to be of particular importance because the mutation at this position alone was sufficient to induce this phenotype. In the present work we have studied the functional and structural consequences of the Phe-92 → Ala mutation in human liver calmodulin. We found that the mutant (CaMF92A) is incapable of activating phosphodiesterase, and the maximal activation of calcineurin is reduced by 40% as compared with the wild type CaM. Impaired regulatory properties of CaMF92A are accompanied by an increase in affinity for Ca$^{2+}$ at the C-terminal domain. To investigate the structural consequences of the F92A mutation, we constructed four recombinant C-terminal domain fragments (C-CaM) of calmodulin (residues 78–148); 1) wild type C-CaMW; 2) Ala substituted for Phe (C-CaMF92A); 3) cysteine residues introduced at position 85 and 112 to lock the domain with a disulfide bond in the Ca$^{2+}$-free (closed) conformation (C-CaM85/112); and 4) mutations 2 and 3 combined (C-CaM85/112F92A). The Cys-containing mutants readily form intramolecular disulfide bonds regardless whether Phe or Ala is present at position 92. The F92A mutation causes a decrease in stability of the domain in the absence of Ca$^{2+}$ as indicated by an 11.8 °C shift in the far UV circular dichroism thermal unfolding curve. This effect is reversed by the disulfide bond in the C-CaM85/112F92A mutant. The C-CaMW peptide shows a characteristic Ca$^{2+}$-dependent increase in solvent-exposed hydrophobic surface which was monitored by an increase in the fluorescence of the hydrophobic probe 1,1'-bis(4-anilino)naphtalene-5,5'-disulfonic acid. The fluorescence increase induced by C-CaMF92A is ~45% lower than that induced by C-CaMW suggesting that the F92A mutation causes a decrease in the accessibility of several hydrophobic side chains in the C-terminal domain of CaM in the presence of Ca$^{2+}$. The Cys-85-Cys-112 disulfide bond causes a 10- or 5.9-fold decrease in Ca$^{2+}$ affinity depending on whether Phe or Ala is present at position 92, respectively, suggesting that coupling between Ca$^{2+}$ binding and the conformational transition is weaker in the absence of the phenyl ring at position 92. Our results indicate that Phe-92 makes an important contribution to the Ca$^{2+}$-induced transition in the C-terminal domain of CaM. This is most likely the reason for the severely impaired regulatory properties of the CaM mutants having Ala substituted for Phe-92.

Calmodulin is the primary intracellular Ca$^{2+}$ receptor responsible for regulation of a large number of enzymes in all eukaryotic cells (2, 3). The crystal structure of CaM from various species (4–7) reveals a dumbbell-shaped molecule with two globular domains linked by a long flexible solvent-exposed helix. Each domain contains two Ca$^{2+}$-binding sites, the helix-loop-helix motifs (termed EF-hands). The C-terminal domain binds Ca$^{2+}$ with high affinity ($K_d = 10^{-6}$ M) and the N-terminal with lower affinity ($K_d = 10^{-4}$ M) (8). Ca$^{2+}$ binding causes exposure of two hydrophobic patches, one in each domain (the target binding sites). This is the key event in the transduction of the Ca$^{2+}$ signal. Recently, the structure of Ca$^{2+}$-free CaM has been solved by multidimensional heteronuclear magnetic resonance techniques (9–11). A comparison between the Ca$^{2+}$-free and Ca$^{2+}$-bound CaM structures shows that the Ca$^{2+}$-induced conformational transition requires a change in the interhelical angle in each of the two globular domains of CaM. In the Ca$^{2+}$-free state the two helices in each EF-hand are almost antiparallel, they are perpendicular to each other, in the Ca$^{2+}$-bound conformation. The transition between the two conformations has been first modeled by Herzberg et al. (12) for the N-terminal domain of TnC, and it is referred to as the HMJ model. We have shown previously that this transition can be blocked with a disulfide bond between Cys residues introduced at specific sites by site-directed mutations leading to a loss of regulatory properties and a decrease in affinity for Ca$^{2+}$ in both TnC (13) and CaM (14).

Although the HMJ model provides a phenomenological description of the Ca$^{2+}$-induced conformational change, the molecular nature of this process is unclear. In particular, it is not known what are the structural elements in the Ca$^{2+}$-dependent regulatory proteins TnC and CaM that are responsible for a tight coupling between Ca$^{2+}$ binding at the Ca$^{2+}$-chelating loops and the subsequent opening of the structure. It is perplexing that some seemingly very similar proteins, members of the EF-hand protein family, do not exhibit this mechanism. For

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1 The abbreviations used are: CaM, calmodulin; CaMF92A, mutant of human liver calmodulin having Ala substituted for Phe-92; C-CaMW, C-terminal domain fragment of human liver calmodulin comprising residues 78–148 equivalent to the tryptic fragment TR$_C$ (1); C-CaMF92A, C-CaM with Ala substituted for Phe-92; C-CaM85/112, C-CaM with Cys substituted for Ile-85 and Leu-112; C-CaM85/112F92A, C-CaM with Ala substituted for Phe-92 and Cys substituted for Ile-85 and Leu-112; TnC, calcium binding component of troponin; DTT, dithiothreitol; smM13, calmodulin binding region of smooth muscle myosin light chain kinase; smM13N, N-terminal 13 amino acids of smM13; PAGE, polyacrylamide gel electrophoresis; bisANS, 1,1'-bis(4-anilino)naphtalene-5,5'-disulfonic acid.
example in calbindin D$_{28K}$, the smallest member of the calmodulin superfamily consisting of 75 amino acids, Ca$^{2+}$ binding causes a slight rotation of the helical segments without a change in the interhelical angles and does not lead to the exposure of hydrophobic sites (15). A comparison of the structures of Ca$^{2+}$-free and Ca$^{2+}$-filled domains in TnC and CaM suggests that the highly conserved Phe residue immediately preceding the first ligand in the two EF-hand domains may have some specific contribution to the conformational change. This residue (Phe-19 and Phe-92 in CaM) undergoes a transition from a position in which half of the phenyl ring is exposed to the hydrophilic core of the protein, with partial exposure of only the C$_8$ atoms (4) which contribute to the target binding hydrophobic site in the Ca$^{2+}$-occupied form (16, 17). This transition is accompanied by a change in polypeptide backbone folding at the last two residues in helix A and E in CaM from a 3$_1$ helix to an $\alpha$-helix in the Ca$^{2+}$-free and Ca$^{2+}$-bound conformation, respectively (9–11). Recent work by Ohya and Botstein on yeast has shown that one of these residues, the Phe-92 in the C-terminal domain of CaM, is critical for the functional properties of this protein (18, 19). Ohya and Botstein examined 14 temperature-sensitive yeast mutants bearing one or more Phe to Ala substitutions in the single essential calmodulin gene of yeast. They found four groups of mutations each showing different characteristic functional defects in actin organization, calmodulin localization, nuclear division, or bud emergence. One of the temperature-sensitive mutants contained a single Phe to Ala mutation at position 92.

The aim of our work was to evaluate the contribution of Phe-92 to the functional and structural properties of CaM. We have substituted Ala for Phe-92 in human liver CaM and found that the mutant (CaM(F92A) is incapable of activating phosphodiesterase, and its ability to activate calcineurin is decreased by 40%. We used the C-terminal domain fragment of CaM corresponding to the TR$_C$ peptide (residues 78–148) to analyze the structural consequences of the F92A mutation alone and in combination with another mutation designed to lock the domain in the Ca$^{2+}$-free conformation. We found that the F92A mutation causes a decrease in stability of the domain in the absence of Ca$^{2+}$, an increase in affinity for Ca$^{2+}$, and a decrease in the ability to interact with a hydrophobic probe bisANS and with a synthetic target peptide. Our results indicate that Phe-92 makes an important contribution to the Ca$^{2+}$-induced conformational transition in the C-terminal domain of CaM. This is most likely the reason for the severely impaired regulatory properties of the mutants having Ala substituted for Phe-92 in yeast and human CaM.

MATERIALS AND METHODS

Protein Mutagenesis Expression and Purification—The DNA (generous gift of Dr. Richard Perham, Cambridge, U.K.) from human liver CaM (20) subcloned between NdeI and PstI restriction sites in pEAD4 (a T7 type expression vector, generous gift of Dr. Don S. Dafering, Whitehead Institute, Cambridge, MA) was used for the expression of wild type CaM and all the mutants. Wild type CaM was expressed and purified as described previously (14). The CaM(F92A) mutant was obtained by substitution of GCA (Ala) codon for the TTT codon of Phe-92 using polymerase chain reaction with appropriate primers (21, 22). For overexpression and purification of CaM(F92A), we followed the same procedure as for the wild type CaM (14).

The C-terminal half-molecule fragment of calmodulin (C-CaMW) has been produced by introducing into the CaM cDNA an NdeI restriction site (CATATG) at residues 76–77. The cDNA fragment corresponding to C-CaMW was amplified using polymerase chain reaction (23), then purified, digested with NdeI and PstI, and ligated into a T7 expression vector pEAD4. The same primers and procedures were used to obtain C-CaM(F92A) and C-CaM85/112 using the CaM(F92A) cDNA (this work) and CaM85/112 cDNA (14), respectively. The triple mutant C-CaM85/112F92A was obtained from C-CaM85/112 cDNA (GCA) for Phe-92 (TTT). All mutations were achieved using polymerase chain reaction with appropriate primers (21, 22) and then ligated into pAE4 vector. The nucleotide sequence of the coding region for each construct was confirmed by dye-terminucleotide sequencing (24) using the Sequenase kit (U. S. Biochemical Corp.). To obtain the cloned cDNA translated, total cDNA was incubated for 4 to 10 hr with a Luria-Bertani broth (LB) medium and grown at 37°C until mid-log phase. Isopropyl-1-thio-β-D-galactopyranoside was then added at 0.5 mM, and the culture was grown at 37°C for 135 min. The cells were then harvested by low speed centrifugation.

Purification of the protein was achieved by freeze-thaw of the low speed pellets with a solution containing 50 mM Tris- HCl, pH 7.5, 2 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride (60 ml/liter of bacterial culture). The extract was centrifuged at 100,000 × g for 30 min; the supernatant was then made up to 0.1 M NaCl and loaded onto a DE52-cellulose (Whatman) column (30 × 3 cm) equilibrated with a solution containing 50 mM Tris- HCl, pH 7.5, 0.1 M NaCl, 1 M CaCl$_2$ (also 2 mM DTT for C-CaM85/112 and C-CaM85/112F92A). The column was washed with the same solution until absorbance of eluent reached baseline level. Protein was then eluted by a 0.1–0.3 M NaCl linear gradient (2 × 500 ml) in a solution containing 50 mM Tris- HCl, pH 7.5, and 1 mM CaCl$_2$. The fractions containing the CaM or CaM fragments (assessed by SDS-PAGE) were pooled. The further purification steps were different for different mutants.

For purification of C-CaMW and C-CaM85/112F92A this combined fractions from the DE52-cellulose column were made to 1 M NaCl and loaded onto a phenyl-Sepharose CL-4B (Pharmacia Biotech) column (9 × 3 cm) equilibrated with a solution containing 50 mM Tris- HCl, pH 7.5, 1 M NaCl, 1 M CaCl$_2$ (also 2 mM DTT for C-CaM85/112). The column was washed with this solution until absorbance of eluent reached baseline level. The protein was then eluted with a solution containing 50 mM Tris- HCl, pH 7.5, 1 M NaCl, 2 mM EGTA (also 2 mM DTT for C-CaM85/112). Fractions eluted with EGTA were pooled, dialyzed into H$_2$O, and lyophilized.

Since C-CaMF92A and C-CaM85/112F92A show a significantly decreased binding to phenyl-Sepharose, further purification of these peptides achieved by precipitation of their corresponding fractions from the DE52-cellulose column, with 8.3% trichloroacetic acid. The precipitate was ultracentrifuged at 100,000 × g for 30 min at 5°C. The pellet was resuspended in 10 ml of a solution containing 50 mM NH$_4$HCO$_3$ and 1 M CaCl$_2$, and the solution was ultracentrifuged again at 100,000 × g for 30 min at 5°C. The supernatant was then passed through a Sephadex G-200 gel filtration column (100 × 1.5 cm) equilibrated with a solution containing 50 mM NH$_4$HCO$_3$ and 1 M CaCl$_2$. Fractions containing the peptides as assessed by SDS-PAGE were pooled, dialyzed into H$_2$O, and lyophilized.

To ensure that C-CaM85/112 and C-CaM85/112F92A were fully oxidized, they were incubated at 25°C at a concentration of 1 mg/ml in a solution containing 4 M urea, 50 mM Tris- HCl, pH 7.5, 0.1 M NaCl, 2 mM EGTA, and 30 mM of each mutant (final concentration) for 36 h, followed by dialysis against a solution containing 50 mM Tris- HCl, pH 7.5, 0.1 M NaCl, 2 mM EGTA. Then 5,5’-dithiobis(2-nitrobenzoic acid) at half the concentration of the SH groups was added to the protein solution, and after 1 h incubation at room temperature, the samples were dialyzed against 4 liters of a solution containing 50 mM Tris- HCl, pH 7.5, 0.1 M NaCl, 36 h with 3 changes of buffer. The proteins were then dialyzed into H$_2$O and lyophilized.

Protein concentration was estimated from UV absorbance using A$_{276}$ (0.1%, 1 cm) = 0.18 for CaM and A$_{276}$ (0.1%, 1 cm) = 0.24 for the CaM mutants. The purity of the mutant proteins was examined by SDS-PAGE gels. The C-terminal domain mutants were also analyzed by electrospray ionization mass spectrometry (25) performed on a Finnigan TSQ700 triple quadrupole mass spectrometer at Harvard Microchem (Cambridge, MA).

Enzyme Purification and Activity Assays—Purification of phenoladiesterase from bovine brain and the measurements of the Ca$^{2+}$-dependent activity was performed according to Ref. 26. For purification of calmodineurin the procedure of Klee et al. (27) was used, and the activity was measured colorimetrically using p-nitrophenyl phosphate as substrate (28). Concentrations of phosphodiesterase and calmodineurin were estimated by densitometric scanning of SDS-PAGE gels using bovine serum albumin as a standard.

bisANS Binding—Increase in bisANS fluorescence was measured for the CaM mutants on a SPEX Fluorolog 2/2/2 photon counting spectrotitrometer (Edison, NJ). 15 µM of each mutant (final concentration)
was added, from a concentrated stock solution, to a quartz cuvette containing 0.8 ml of a solution containing 5 \( \mu \)M bisANS, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM EGTA. An emission scan was taken from 400 to 600 nm with excitation at 394 nm, and then 2 mM CaCl\(_2\) was added, and the scan was repeated.

Ca\(^{2+}\) Titrations—Ca\(^{2+}\) binding to the C-CaM mutants was monitored by tyrosine fluorescence. Titrations were carried out in a solution containing 10 \( \mu \)M protein, 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM nitriilotriacetic acid as described previously (13). Measurements were performed at 25 °C with a SPEX Fluorolog 2/2/2 photon counting spectrophotometer (Edison, N J) in 1-cm cuvette. Concentrations of free Ca\(^{2+}\) were calculated according to Ref. 29 using published stability constants (30). Data points were fitted with the Hill equation, \( F = F_0 + \Delta F (K_{CaM} + [Ca^{2+}])/(1 + (K_{CaM} + [Ca^{2+}])\) using the Levenberg-Marquardt algorithm as implemented by Kaleidagraph program from Ablebeck Software. \( F_0 \) and \( \Delta F \) is the initial fluorescence and maximal change in fluorescence, respectively, \( K_{CaM} \) is the apparent binding constant, and \( n \) is the Hill coefficient.

Mean Residue Ellipticity Measurements—Measurements were performed on an AVIV (Lakewood, N J) 62A DS Circular Dichroism Spectrometer. Ellipticity values from 250 to 200 nm were recorded for all proteins (0.25 mg/ml) in a solution containing 15 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.2 mM EGTA. 0.5 mM CaCl\(_2\) was then added and the scan repeated. Protein concentration was calculated from amino acid analysis. Mean residue ellipticity values were then calculated from ellipticity values at 222 nm = Ca\(^{2+}\).

Thermal Unfolding—Ellipticity values at 222 nm were recorded for samples containing 0.2 mg/ml protein, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.2 mM EGTA as a function of temperature in 0.5 °C steps with 24-s equilibration time and 5-s averaging time. The denaturation curves for the C-CaM mutants were analyzed assuming a two-state equilibrium between native (n) and unfolded (u) forms of the protein with the temperature-dependent equilibrium constant \( K \) as described previously (31, 32). The data were fitted using the nonlinear least-squares procedure (33).

Target Peptide Binding—A synthetic peptide (smM13N) corresponding to the N-terminal part of the CaM binding region of smooth muscle myosin light chain kinase (residues 494–513) (34, 35) was used as a target for binding to C-CaM mutants. The smM13N peptide having the amino acid sequence ARRWKOKTHGAVR was synthesized using an automatic solid phase peptide synthesizer (Applied Biosystems 431A) and purified by high performance liquid chromatography. Increasing amounts of C-CaM mutants were added to 5 \( \mu \)M smM13N in a solution containing 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.2 mM CaCl\(_2\). The binding was monitored by Trp fluorescence measured at 320 nm upon excitation at 295 nm. Titration data were fitted assuming that the change in Trp fluorescence is proportional to the fraction of sites occupied (n): \( F = F_0 + \Delta F \cdot n / B \cdot (1 + (K_{CaM} + [Ca^{2+}])\) where \( K_{CaM} \) is the apparent equilibrium binding constant, \( B \) is the total concentration of the C-CaM binding sites, \( n \) is the apparent stoichiometry (36). Data were fitted using Kaleidagraph program (Ablebeck Software).

RESULTS

The purpose of these studies was to evaluate the functional and structural role of Phe-92 in CaM. We have tested the ability of CaMF92A to activate phosphodiesterase and calcineurin. Fig. 1 shows the enzymatic activity of phosphodiesterase (A) and calcineurin (B) as a function of concentration of the wild type CaM and the CaMF92A mutant. CaMF92A fails to activate phosphodiesterase even at micromolar concentrations, whereas nanomolar concentrations of the wild type CaM are sufficient for activation. In the case of calcineurin the maximal activation with CaM92A is approximately 40% lower than that with the wild type CaM. The transition midpoints of calcineurin activation curves for both CaM and CaM92A appear to be similar. However, this cannot be taken as an indication of their similar affinities for this enzyme because the enzyme concentration in the assay was above the \( K_m \). Such concentration was necessary due to a relatively low sensitivity of our assay utilizing phosphonitrophenol as substrate.

Calcium titrations of the C-terminal domain of CaMF92A monitored with tyrosine fluorescence have shown a shift of the transition midpoint to lower Ca\(^{2+}\) concentrations indicating an increase in the affinity for Ca\(^{2+}\) as compared with the wild type protein (data not shown). A similar effect of the F92A mutation was observed for the C-terminal domain fragment (see below). It is plausible that the impaired regulatory properties of CaMF92A result from the missing contribution of the phenyl ring at position 92 to the binding/activation of the target en-
zymes. However, the increase in Ca$^{2+}$ affinity suggests that some structural effects may be responsible. The C-terminal half-molecule mutants were used to test this hypothesis.

Electrophoretic Mobility of the C-CaM Mutants—A comparison of electrophoretic mobilities of C-CaM mutants on polyacrylamide gels in the presence and absence of urea and Ca$^{2+}$ is shown in Fig. 2. In the presence of Ca$^{2+}$ (Fig. 2, B and D) all the mutants have similar mobility consistent with their almost identical molecular weight and charge. In contrast there are large differences in electrophoretic mobility of these proteins on the gels run in the presence of EDTA (Fig. 2, A and C). In particular, there is a DTT-dependent decrease in mobility of C-CaM85/112 and C-CaM85/112F92A indicating that, in the absence of reducing agents, these peptides contain intramolecular disulfide bonds (Fig. 2A). The higher mobility of the disulfide cross-linked forms of C-CaM85/112 and C-CaM85/112F92A is most likely due to their increased resistance to urea-induced unfolding. The differences in mobility in the absence of urea (Fig. 2C) appear to reflect differences in the folding of the peptides. C-CaMW has the same mobility as the disulfide-containing mutants indicating similar apparent molecular size. In contrast C-CaMF92A and the reduced forms of C-CaM85/112 and C-CaM85/112F92A have much lower electrophoretic mobility, most likely due to their lower structural stability (see below).

Secondary Structure and Stability of C-CaM Mutants—Table I shows the mean residue ellipticity ($\Theta_{222}$) of the C-CaM mutants at 25 °C in the presence and absence of Ca$^{2+}$. C-CaM85/112 and C-CaM85/112F92A exhibit a slightly lower Ca$^{2+}$-dependent increase in mean residue ellipticity as compared with C-CaMW, whereas C-CaMF92A shows a significantly larger Ca$^{2+}$-dependent increase in $\Theta_{222}$ due to a lower $\alpha$-helix content in the absence of Ca$^{2+}$.

We have compared the structural stability of the mutants in the presence of EGTA by recording ellipticity at 222 nm as a function of temperature. All peptides undergo cooperative heat-induced unfolding with a well defined transition (Fig. 3) characterized by $T_m$, the temperature at which half of the molecules are unfolded, the enthalpy of unfolding $\Delta H_m$ (at the temperature $T_m$) and heat capacity change $\Delta C_p$ (31). Substitution of Ala for Phe-92 causes a decrease in stability of the peptide in the absence of Ca$^{2+}$ as evidenced by an 11.8 °C decrease in $T_m$ (Fig. 3 and Table II). This effect is reversed by the Cys-85–Cys-112 disulfide bond in C-CaM85/112F92A. The computer fit of the data for the DTT-reduced C-CaM85/112F92A resulted in an obviously incorrect $T_m$ value and a much larger value of $\Delta C_p$ than those obtained for other peptides. This was apparently due to a poorly defined initial slope of the unfolding curve. To correct for this error a fixed value for the initial slope (an average of those obtained for other mutants) was used in the calculations. This resulted in stable values of $T_m$, $\Delta H_m$, and $\Delta C_p$ (Table II). It is somewhat surprising that the disulfide bond between Cys-85 and -112 does not increase the stability of the C-terminal domain of CaM except when Ala is present at position 92. This is in contrast to the N-terminal domain of TnC in which a disulfide bond caused a large increase in stability (37). In the presence of Ca$^{2+}$, the structural stability of all the C-CaM mutants was much higher, and no cooperative unfolding was observed up to 90 °C.

Ca$^{2+}$ Binding—We used tyrosine fluorescence intensity to monitor the Ca$^{2+}$ binding to C-CaM peptides (Fig. 4). C-CaMW exhibits a characteristically high Ca$^{2+}$ affinity with a $K_a$ of 9.7 \times 10^9 M^{-1} and a Hill coefficient of 1.5 (Table III). The substitution of Ala for Phe causes a 2-fold increase in Ca$^{2+}$ affinity resulting in a $K_a$ of 1.9 \times 10^9 M^{-1} and a Hill coefficient of 1.8. The Cys-85–Cys-112 disulfide bond causes a 10-fold decrease or 5.9-fold decrease in Ca$^{2+}$ affinity depending on whether Phe or Ala is present at position 92 (Table III). Ca$^{2+}$ binding to both C-CaM85/112 and C-CaM85/112F92A is noncooperative (Hill coefficient n = 1.0). Both the decrease in affinity and the loss of cooperativity in the disulfide cross-linked mutants are consist-

**TABLE I**

Mean residue ellipticity ($\Theta_{222}$) of C-CaM mutants

| Mutant          | + EGTA ($\Theta_{222}$) | + Ca$^{2+}$ | $\Delta$ (deg cm$^2$ dmol$^{-1}$) |
|-----------------|-------------------------|-------------|----------------------------------|
| C-CaMW          | 13,500                  | 16,900      | 1.25                             |
| C-CaM85/112     | 13,800                  | 15,400      | 1.11                             |
| C-CaMF92A       | 12,700                  | 16,500      | 1.30                             |
| C-CaM85/112F92A| 14,700                  | 16,600      | 1.13                             |
ent with the HMJ model as discussed previously (13, 14).

Interaction with a Hydrophobic Probe bisANS—We have studied the exposure of hydrophobic sites in the C-CaM mutants in the presence of Ca\(^{2+}\) by monitoring their ability to increase the fluorescence intensity of the hydrophobic probe bisANS (Fig. 5). The titration curves in Fig. 5A indicate that the peptides bind bisANS with a very low affinity and undetermined stoichiometry. The fluorescence of bisANS increases monotonically with increasing concentrations of the peptides and even at 100 \(\mu\)M does not reach saturation. Thus, at the peptide concentrations used in our experiments (15 \(\mu\)M) the fluorescence intensity is proportional to the amount of bisANS bound to the protein, and it can be taken as a relative measure of the protein's hydrophobicity.

C-CaMW induces a characteristic large Ca\(^{2+}\)-dependent increase in bisANS fluorescence (Fig. 5B). In contrast, the fluorescence increase induced by the disulfide cross-linked mutants C-CaM85/112 and C-CaM85/112F92A is very small (Fig. 5B) but becomes similar to that induced by CaMW upon reduction with DTT (data not shown). It is interesting that the Ca\(^{2+}\)-induced increase in the solvent-exposed hydrophobic surface in C-CaM (513 \(\AA^2\)) represents only 18% of the total hydrophobic surface in this domain (Table IV). However, the residues exposed upon Ca\(^{2+}\) binding form a continuous hydrophobic cluster having a characteristic concave shape which is apparently necessary for the binding of bisANS or the target peptide/enzyme (for review see Refs. 38 and 39). It is clear that this characteristic property is reversibly abolished by the Cys-85–Cys-112 disulfide bond. Most remarkably the Ca\(^{2+}\)-dependent characteristic property is reversibly abolished by the Cys-85–Cys-112 disulfide bond. Most remarkably the Ca\(^{2+}\)-dependent characteristic property is reversibly abolished by the Cys-85–Cys-112 disulfide bond.

Fig. 4. Calcium titrations of C-CaM mutants. Calcium binding was monitored by Tyr fluorescence (\(\lambda_{em} = 280\) nm; \(\lambda_{ex} = 304\) nm). Each sample contained 5 \(\mu\)M protein, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EGTA, and 1 mM nitritotriacetic acid. Each data point is an average from three titrations. Error bars represent the standard deviation, and the solid lines represent the fit using the Hill equation. Parameters of the fit are given in Table III.

Fig. 5. Interaction of bisANS with C-CaM mutants. A, titrations of bisANS (5 \(\mu\)M bisANS in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EGTA 2 mM CaCl\(_2\)) with C-CaM mutants. B, calcium-dependent increase in fluorescence of bisANS induced by C-CaM mutants. 15 \(\mu\)M of each mutant was added to 5 \(\mu\)M bisANS in a solution containing 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM EGTA. An emission scan was taken from 400 to 600 nm (1-nm intervals) at an excitation wavelength of 394 nm. Then 2 mM CaCl\(_2\) was added, and the scan was repeated. The difference between the two scans is shown. For clarity data points at 4-nm intervals are shown only. •, C-CaMW; ●, C-CaM85/112; ○, C-CaMF92A; □, C-CaM85/112F92A.

### Table II

Thermodynamic parameters of the thermal unfolding of C-CaM mutants in the presence of EGTA

\(\Delta T_m\) is the temperature at which half of the molecules are unfolded, \(\Delta H_m\) and \(\Delta C_p\) are the enthalpy of unfolding and heat capacity change, respectively, at the temperature \(T_m\) (31).

| C-CaMW | G-CaM85/112 | C-CaMF92A | C-CaM85/112F92A |
|--------|-------------|-----------|-----------------|
| 57.9 ± 0.6 | 60.1 ± 1.0 | 46.1 ± 0.6 | 58.7 ± 0.6 |
| 30.6 ± 1.2 | 24.1 ± 1.1 | 23.0 ± 1.1 | 29.6 ± 1.0 |
| 362 ± 183 | 622 ± 86 | 532 ± 80 | 609 ± 133 |

### Table III

Fitting parameters of the \(Ca^{2+}\) titration curves

| Protein | \(K_a\) (M\(^{-1}\)) | Hill coefficient |
|---------|------------------|------------------|
| C-CaMW | \(9.7 \times 10^6\) | 1.5 |
| C-CaMF92A | \(1.9 \times 10^6\) | 1.8 |
| C-CaM85/112F92A | \(3.2 \times 10^6\) | 1.0 |
for C-CaM F92A indicates that the mutation must have affected many other hydrophobic side chains in this protein.

Interaction with a Target Peptide—We have assessed the effects of the various mutations introduced into C-CaM on the binding to a synthetic target peptide designed on the basis of the CaM binding region of smooth muscle myosin light chain kinase (smM13). The high resolution structure of the CaM-smM13 complex shows that the N-terminal segment of smM13 binds to the C-terminal domain of CaM and vice versa (16, 17). For our studies we have synthesized a peptide corresponding to the N-terminal part of smM13 (ARRKWQKTGHAVR). Fig. 6 shows the binding of smM13N (5 μM) to C-CaM mutants measured by an increase in Trp fluorescence (the 5th residue in smM13N). C-CaMW induces a 6-fold increase in Trp fluorescence of smM13N. The effect of C-CaMF92A is significantly smaller. Fitting of the data in Fig. 6 gave the apparent association constants of 4.1 × 10^4 M^{-1} and 3.6 × 10^4 M^{-1} for C-CaMW and C-CaMF92A, respectively. Thus, the F92A mutation causes more than a 10-fold decrease in affinity for smM13N but does not abolish the binding. Both C-CaM/112 and C-CaM85/112F92A cause essentially no increase in Trp fluorescence of smM13N suggesting that these mutants do not bind smM13N to any significant extent. This is consistent with the bisANS binding data and provides further evidence that the disulfide bond makes the interaction site inaccessible.

**DISCUSSION**

Our current work on human liver CaM shows the vital importance of the Phe-92 residue for the regulatory properties of CaM consistent with the observations of Ohya and Botstein (18, 19) on yeast CaM. It is remarkable that a single point mutation has such a dramatic effect on the regulatory properties of CaM. The analysis of the three-dimensional structure of CaM indicates that Phe-92 is involved in numerous interactions in the hydrophobic core of the C-terminal domain (4–7). Phe-92 also contributes to the target interactions in the CaM-smM13 complex (16, 17, 40). Both these properties are shared by several other Phe residues in CaM, but a systematic analysis of F → A mutations at all positions in yeast CaM has shown that the F92A mutation was the only one which alone could impart a temperature-sensitive phenotype (18, 19), clearly indicating a unique role for this residue. An explanation for this phenomenon stemming from our data is that Phe-92 has an important structural function and the mutation affects many other residues interfering with their optimal contribution to the CaM target interaction/activation.

The Ca^{2+}-dependent interaction of CaM with hydrophobic probes such as bisANS is interpreted to represent the exposure of hydrophobic residues, the target binding sites. The bisANS was first introduced by Rosen and Weber (41) and became a popular probe to study hydrophobic properties of proteins. We have utilized the increase in fluorescence intensity of bisANS upon binding to C-CaM and its mutants to provide a relative measure of hydrophobic surface exposure. C-CaM85/112 and C-CaM85/112F92A in the oxidized form cause little increase in the bisANS fluorescence due to the disulfide bridge blocking the access of the probe to the hydrophobic residues regardless of the presence of Ca^{2+}. Most importantly, the fluorescence increase caused by the C-CaMF92A mutant is approximately 45% lower than that induced by C-CaMW. This indicates a significant decrease in available hydrophobic sites for the binding of bisANS which is opposite to what one would expect based on theoretical calculations. It could be argued that having one less hydrophobic residue (Ala replaced Phe) could result in a decrease in the hydrophobicity of the site causing a reduction in the bisANS fluorescence in the absence of any alterations of the structure. However, the phenyl ring of Phe-92 accounts for only a small fraction of the hydrophobic surface in C-CaMW. In fact, calculations of the solvent-exposed surface area (in the presence of Ca^{2+}) show a slightly larger exposure of hydrophobic surface in C-CaM85/112F92A (2837 Å^2) than in the wild type protein (2812 Å^2). Thus, the Phe-92 → Ala mutation must cause a decrease in the exposure of other hydrophobic residues. Such an effect would be consistent with an incomplete opening of the domain upon Ca^{2+} binding. Similarly, the large decrease in the affinity of smM13N peptide for C-CaMF92A, as compared with the wild type protein, can be interpreted as resulting from incorrect conformation of the binding site rather than from the lack of an important contact site (the phenyl ring of Phe-92).

The two types of mutations used in this study have opposite effects on Ca^{2+} binding. The large decrease in Ca^{2+} affinity caused by the disulfide bond results from the coupling between the binding of Ca^{2+} to the Ca^{2+}-chelating loop and the subsequent conformational transition which is the hallmark of the Ca^{2+}-dependent regulation. When the opening of the structure is blocked with a disulfide bond then the coupling mechanism prevents the Ca^{2+}-binding loop from attaining the correct geometry, which results in a decrease in Ca^{2+} affinity. It is less clear why the removal of the phenyl ring at position 92 should cause an increase in Ca^{2+} affinity. It appears that two different mechanisms could explain such an effect, each ascribing a different role to Phe-92.

One possibility is that the hydrophobic phenyl ring of Phe-92

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**TABLE IV**

| Hydrophobic area | −Ca^{2+} | +Ca^{2+} |
|------------------|----------|----------|
| Total hydrophobic area | 2299 | 2812 |
| Phe-92 contribution | 14 | 11 |
| Change upon F92A mutation | +13 | +25 |

* a Contribution from side chain carbon atoms only.
contributes to the stability of the Ca$^{2+}$-free conformation of CaM, in particular to the interhelical interactions that keep the protein in the closed conformation. The free energy of Ca$^{2+}$ interaction with its ligands, viz. carboxyl and carbonyl groups of the loop, is used in part for breaking the interhelical interactions. Thus, a protein modification that decreases such interactions would result in an increase in apparent Ca$^{2+}$ binding constant. The lower unfolding temperature of C-CaMF92A (in the absence of Ca$^{2+}$) appears to support this hypothesis. However, in such a case no difference in Ca$^{2+}$ affinity between C-CaM85/112 and C-CaM85/112F92A should be observed. Since there is no opening of the structure upon Ca$^{2+}$ binding in these mutants, differences in interhelical interactions in the Ca$^{2+}$-free conformation should not play any role. This is clearly not the case. In fact the F92A mutation causes a larger increase in Ca$^{2+}$ affinity in the presence of the disulfide bond (3.3-fold increase) than in its absence (2.0-fold increase).

The effect of the F92A mutation may also be interpreted on the assumption that Phe-92 contributes to the coupling between Ca$^{2+}$ binding at the loop and the conformational transition. This would mean that in the absence of Phe-92 the ligands at the Ca$^{2+}$ binding loop can interact effectively with Ca$^{2+}$ even in the absence of or only after partial opening of the structure. In such a case a smaller fraction of the Ca$^{2+}$-binding free energy would be used for the conformational transition since fewer hydrophobic interhelical interactions would have to be broken. Moreover, in the Ca$^{2+}$-bound conformation a smaller hydrophobic surface would be exposed to solvent. Both factors would cause an increase in the apparent Ca$^{2+}$ binding constant. However, the accessibility of the target binding hydrophobic surface would be decreased. Our observations that F92A mutation causes an increase in Ca$^{2+}$ affinity and a decrease in bisANS binding and in affinity between CaM and Ca$^{2+}$ should have a smaller effect on the Ca$^{2+}$ affinity than in its absence (2.0-fold increase).

In conclusion our results indicate that Phe-92, the highly conserved phenylalanine residue at the position immediately preceding the first Ca$^{2+}$ ligand in the C-terminal domain of CaM, makes an important contribution to the Ca$^{2+}$-induced conformational transition. This residue appears to be involved in the coupling between Ca$^{2+}$ binding at the loop and the opening of the structure.
The Role of Phe-92 in the Ca-induced Conformational Transition in the C-terminal Domain of Calmodulin
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