THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 22, Issue of June 3, pp. 15546–15552, 1994
Printed in U.S.A.

The Effect of Met → Leu Mutations on Calmodulin's Ability to Activate Cyclic Nucleotide Phosphodiesterase*

(Received for publication, February 15, 1994)

Mingjie Zhang‡, Ming Li‡, Jerry H. Wang§¶, and Hans J. Vogel‡§‖

From the ‡Departments of Biological Sciences and §Medical Biochemistry, The University of Calgary, 2500 University Drive NW, Calgary, Alberta, T2N 1N4, Canada

Calmodulin (CaM) has two hydrophobic surface patches that are particularly rich in Met residues, and these are the major contact areas where CaM interacts with its target enzymes. The amino acid Leu has been introduced by site-directed mutagenesis to replace all the Met residues in CaM. All nine individual Met → Leu mutants of CaM as well as some double and quadruple mutants were expressed in *Escherichia coli*. All mutants could be purified by calcium-dependent hydrophobic affinity chromatography, indicating that they still expose their hydrophobic surfaces upon binding calcium. Each single Met → Leu mutation in the C-terminal domain of the protein had little effect on its ability to activate phosphodiesterase (PDE), while a quadruple mutant with four C-terminal Leu residues instead of Met has a significantly lower affinity for PDE. The M36L mutant is a poor activator compared with the other three N-terminal single Met → Leu mutants, which have a slightly lower affinity for PDE than wild-type CaM. The introduction of a positively charged Arg for Met-145 resulted in an almost complete loss of CaM's ability to activate PDE. Nuclear magnetic resonance spectroscopy was used to show that most CaM mutants retain their overall three-dimensional structure. Thus, the altered activation properties appear to arise from differences in the flexibility and polarizability of the Met and Leu sidechains, rather than from structural perturbations.

Calmodulin (CaM)[1] performs its functional role, which couples the intracellular Ca^{2+} signal to cytosolic biological events, by binding in a calcium-dependent manner to a wide spectrum of intracellular proteins and enzymes (for reviews, see Hiraoki and Vogel (1987) and Means *et al.* (1991)). Upon binding Ca^{2+}, CaM undergoes significant conformational changes and exposes a hydrophobic patch on the surface of each globular domain of the protein (Vogel *et al.*, 1983). X-ray structures of Ca^{2+}-CaM show that the hydrophobic patches on the protein have a crescent shape and consist of the hydrophobic residues Met, Leu, Ile, Phe, and Val (Babu *et al.*, 1988; Chattopadhyaya *et al.*, 1992; Rao *et al.*, 1993). Only the Ca^{2+} form of CaM is capable of binding to and activating its target enzymes. The CaM-binding domains of these enzymes are usually contained in a contiguous stretch of ≈20 amino acid residues. These domains do not show a high amino acid sequence homology with each other. However, most of the CaM-binding domains are hydrophobic and positively charged, and they have a tendency to form amphiphilic helices. It is generally accepted that CaM uses its two hydrophobic surface regions to interact with the hydrophobic face of the amphiphilic helical part of its target enzymes (O'Neil and DeGrado, 1990a).

The two hydrophobic patches on CaM are particularly rich in Met; and the eight Met side chains contribute as much as 46% to the two hydrophobic surface areas of the protein (O'Neil and DeGrado, 1990a). The Met residues are suggested to be the main reason that CaM is capable of binding its target enzymes in a sequence-independent manner (O'Neil and DeGrado, 1990a; Gellman, 1991). A similar sequence-independent interaction with amphiphilic positively charged helices has also been observed between the Met-rich 54-kDa subunit of the signal recognition particle (SRP54) and signal peptides. It is believed that in SRP54, three amphiphilic α-helices are formed, each of which carries several Met residues on its nonpolar face. This forms a Met-rich nonpolar surface that allows SRP54 to interact with apolar signal peptides with diverse sequences (Bernstein *et al.*, 1989). Several ideas have been put forward to answer the question why Met residues can play a special role in the sequence-independent recognition of nonpolar protein surfaces. O'Neil and DeGrado (1990a) and Bernstein *et al.* (1989) have pointed out that the side chain of Met is unbranched and thus can have considerable conformational flexibility compared with the side chains of Leu and Ile. Using butane and 2-thiobutane as model compounds, Gellman (1991) drew attention to the fact that the energy barrier for the rotation around the C_γ-S bond of the Met side chain is much lower (~3 kJ/mol) than that of the C_γ-C_ϵ bond of other aliphatic side chains; this results from the fact that the C-S bond is about 0.3 Å longer than the C-C bond. Consequently, the side chain of Met residues in proteins can adopt more configurations at room temperature, because the energy barriers between different configurations are low compared with the thermal equilibrium energy. Indeed, various configurations of the C_γ-S bound in Met residues have been observed in the crystal structures of proteins (Janin *et al.*, 1978). Thus, a Met-rich apolar surface on a protein is malleable and can adapt itself to binding partners of varying dimensions.

Studies of the association between cyclodextrin and various compounds of different size led Eftink *et al.* (1989) to the conclusion that the London dispersion force is one of the dominant forces governing intermolecular association via nonpolar surface contacts. These authors also pointed out that the London dispersion force is one of the main forces in protein-ligand and protein-protein interactions. A sulfur atom has a much higher polarizability than a carbon atom (Fersht, 1985); hence, the

* This work was financially supported by operating grants from the Medical Research Council (MRC) of Canada (to J. H. W and H. J. V.). The NMR spectrometer used in this study was funded by MRC and by the Alberta Heritage Foundation for Medical Research (AHFMR). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Scientist of the Alberta Heritage Foundation for Medical Research.
‖ Scholar of the Alberta Heritage Foundation for Medical Research. To whom correspondence should be addressed. Tel.: 403-220-6006; Fax: 403-289-9311.

[1] The abbreviations used are: CaM, calmodulin; HMQC, heteronuclear multiple quantum coherence; PDE, cyclic nucleotide phosphodiesterase; PAGE, polyacrylamide gel electrophoresis; wt, wild-type; MOPS, 4-morpholineethanesulfonic acid.

sulfur-atom-containing Met side chain is more polarizable and "sticky" than amino acids made purely of aliphatic carbons. The interaction between CaM and its target enzymes involves extensive nonpolar surface contact; thus, the incorporation of multiple methionine residues should enable the malleable hydrophobic patches on CaM to bind to its target enzymes with high affinity (Gellman, 1991). The polarizable Met residues may also contribute to the formation of the stable solvent accessible hydrophobic surface of Ca^{2+}-CaM by interacting with the highly polar water molecules.

In this work, site-directed mutagenesis has been used to study the importance of the flexibility and polarizability of the individual Met residues in CaM. The Met residues in CaM have been mutated to Leu, and the ability of the mutated CaM samples to activate the enzyme cyclic nucleotide phosphodiesterase (PDE) has been determined. Moreover, several double mutants and a quadruple mutant of CaM have also been generated, and their PDE activation profiles have been studied as well. Not surprisingly, the introduction of a single charged residue into the C-terminal domain of CaM was found to abolish almost completely the function of the protein. In addition, the structural effects of some of the mutations were studied by NMR spectroscopy.

MATERIALS AND METHODS

Unless otherwise stated, all the chemicals used were the products of Sigma. ^{13}C-methyl-Met was purchased from MSD (Montreal, Canada). The 20-residue peptide, Ac-QTEKMWQRLKGILRCLVKQL-NH_2, which correspondences to the amino acid sequence of 22–41 of bovine brain PDE and is known to be the CaM-binding domain of PDE (Charbonneau *et al.*, 1991), was commercially synthesized by the Core Facility for Protein/DNA Chemistry, Queen's University, Canada. The purity of the PDE peptide was >95% as judged by high performance liquid chromatography and amino acid analysis. Bovine brain PDE was purified as described by Sharma *et al.* (1983).

Molecular Cloning and Site-directed Mutagenesis—The reagents used in site-directed mutagenesis of CaM were purchased from Life Technologies, Inc., or from New England Biolabs. Restriction enzyme digests, DNA ligations, and other recombinant DNA techniques were performed essentially as described by Sambrook *et al.* (1989). All the custom oligonucleotides were synthesized on a Gene Assembler Plus DNA synthesizer and were purified following the recommended procedures of the manufacturer. The plasmid pCaM containing a synthetic bovine CaM gene was generously provided by Dr. T. Grundström at the University of Umeå, and it is described in detail elsewhere (Waltersson *et al.*, 1993; Zhang and Vogel, 1993).

The site-specific mutations of individual Met residues in CaM were generated by the polymerase chain reaction technique essentially in the same manner as described previously (Zhang and Vogel, 1993). To convert Met to Leu, the nucleic acid codon ATG of Met was replaced by CTG of Leu in the mutation primers. Single mutants as well as double mutants of Met-71, -72 → Leu and Met-144, -145 → Leu were created by using degenerate mutation primers, *i.e.* degenerate codons (A/C)TG(A/C)TG were used at the mutation sites. Double mutants of Met-109, -124 → Leu CaM and Met-36, -51 → Leu CaM were obtained by using CaM genes with the single point mutations Met-109 → Leu and Met-36 → Leu as polymerase chain reaction templates, respectively. The cassette replacement of the *PstI/ClaI* fragment of M144,145L-CaM gene with the same restriction fragment of M109,124L-CaM gene generated the quadruple mutant of M109,124,144,145L-CaM. Similarly, the M36,51,71,72L-CaM quadruple mutant was created by cassette exchange of the *KpnI/SacI* fragment of M71,72L-CaM with that of M36,51L-CaM. All the mutations were verified by sequencing the proper DNA fragments either in pCaM or pBS^+ plasmids. The mutant CaMs were expressed in MM294 *Escherichia coli* cells grown in L-broth medium as described previously (Zhang and Vogel, 1993) and purified using phenyl-Sepharose hydrophobic chromatography (Vogel *et al.*, 1983; Putkey *et al.*, 1985). Met-methyl-^{13}C selectively labeled wt-CaM and CT-CaM were prepared by growth and expression in minimal MOPS medium as described earlier (Zhang and Vogel, 1994b).

Protein Polyacrylamide Gel Electrophoresis (PAGE)—Denaturing SDS-PAGE (Laemmli, 1970) was used to analyze the CaM mutants. The samples were boiled for 3 min in sample buffer containing either 5 mM $CaCl_2$ or 5 mM EDTA before loading onto 15% gels. No Ca^{2+} or EDTA was added to the gels or running buffer.

Assay of the Activity of Phosphodiesterase—The CaM-dependent activation of PDE was measured following the release of free phosphate as described by Sharma and Wang (1979) with minor modifications, and the details of the experiment have been described previously (Zhang *et al.*, 1994). The CaM stimulation of PDE was 10-fold compared with the basal activity, which was measured in the absence of CaM.

NMR Studies of CaM Mutants—All NMR samples were prepared using D_2O as solvent. The sample pH values are reported as direct readings from a glass electrode; no corrections for solvent isotope effects have been made. The concentration of CaM was ≈1 mM for each sample. The complex of Met-methyl-^{13}C selectively labeled CaM with the PDE peptide at a 1:1 ratio was prepared in the same manner as described previously for the complex of CaM with the CaM-binding domain of myosin light chain kinase (Zhang and Vogel, 1993).

All NMR spectra were acquired on a Bruker AMX500 spectrometer using a 5-mm inverse-detected broadband probe. The solvent D_2O was used as the frequency lock for the spectrometer. For each one-dimensional ^{1}H NMR experiment, 128 scans with a 5500-Hz sweep width and 8-K data points were collected. Two-dimensional (^{1}H,^{13}C)-heteronuclear multiple quantum coherence spectra (HMQC) of Met-methyl-^{13}C selectively labeled CaM and its complex with the PDE peptide were recorded using a published pulse scheme (Bax *et al.*, 1983); the phase-sensitive mode was achieved by using time proportional phase increment technique (Marion and Wüthrich, 1983). The NMR data were processed on an X32 computer using the Bruker Uxnmr software. For two-dimensional (^{1}H,^{13}C)-HMQC spectra, the data were zero filled once and a 72 °C-shifted sine-squared window was applied in both dimensions prior to the Fourier transformation.

RESULTS

All of the individual Met → Leu mutants of CaM as well as the C-terminal double and quadruple mutants could be expressed at a reasonably high level in MM294 *E. coli* cells (20~60 mg/liter in L-broth medium). All these mutant CaMs could be purified using the standard Ca^{2+}-dependent phenyl-Sepharose hydrophobic chromatography (Vogel *et al.*, 1983; Putkey *et al.*, 1985). These results indicate that all individual Met → Leu mutants can undergo the Ca^{2+}-dependent exposure of a hydrophobic surface. However, it is known that the hydrophobic patch in each domain of CaM can interact with phenyl-Sepharose upon binding calcium (Vogel *et al.*, 1983), and, hence, it is possible that binding occurs primarily to the nonmutated domain. For example, the mutant M145R could still be purified in this fashion, presumably because its N-terminal domain could still bind to the phenyl-Sepharose. We have observed much lower expression levels for the double mutants M36,51L and M71,72L, although, again, these two double mutants could be purified by the same purification method used for wild-type CaM. However, the expression of the quadruple mutant M36,51,71,72L-CaM and a single mutant M72R-CaM was unsuccessful. All the purified mutant CaMs show UV-vis spectra similar to wild-type CaM (data not shown).

Electrophoretic Mobility of CaM Mutants in SDS-PAGE—It is well known that CaM migrates differently during SDS-PAGE in the presence of Ca^{2+} or EDTA. The presence of Ca^{2+} will result in faster migration of the protein (Klee *et al.*, 1979), although the basis for this mobility shift is not understood. Fig. 1 shows the SDS-PAGE results for all of the mutant CaMs in the presence of 5 mM $CaCl_2$ or 5 mM EDTA. It is directly seen in the figure that nearly all single-point mutations have little effect on the mobility of the apo-protein. However, some of the single-point mutants showed significant changes in their mobility in the presence of Ca^{2+}. For example, M145L moves significantly faster in the presence of Ca^{2+}, while M36L runs slower. In addition, the M36L mutant protein has a smaller Ca^{2+}-dependent mobility shift. The mobility of M76L in the presence of Ca^{2+} or EDTA is exactly the same as that of wild-type CaM. In contrast to the M36L mutant, the M36,51L double mutant has a normal mobility compared with wt-CaM both in the presence of Ca^{2+} and EDTA, and this may have some cor-

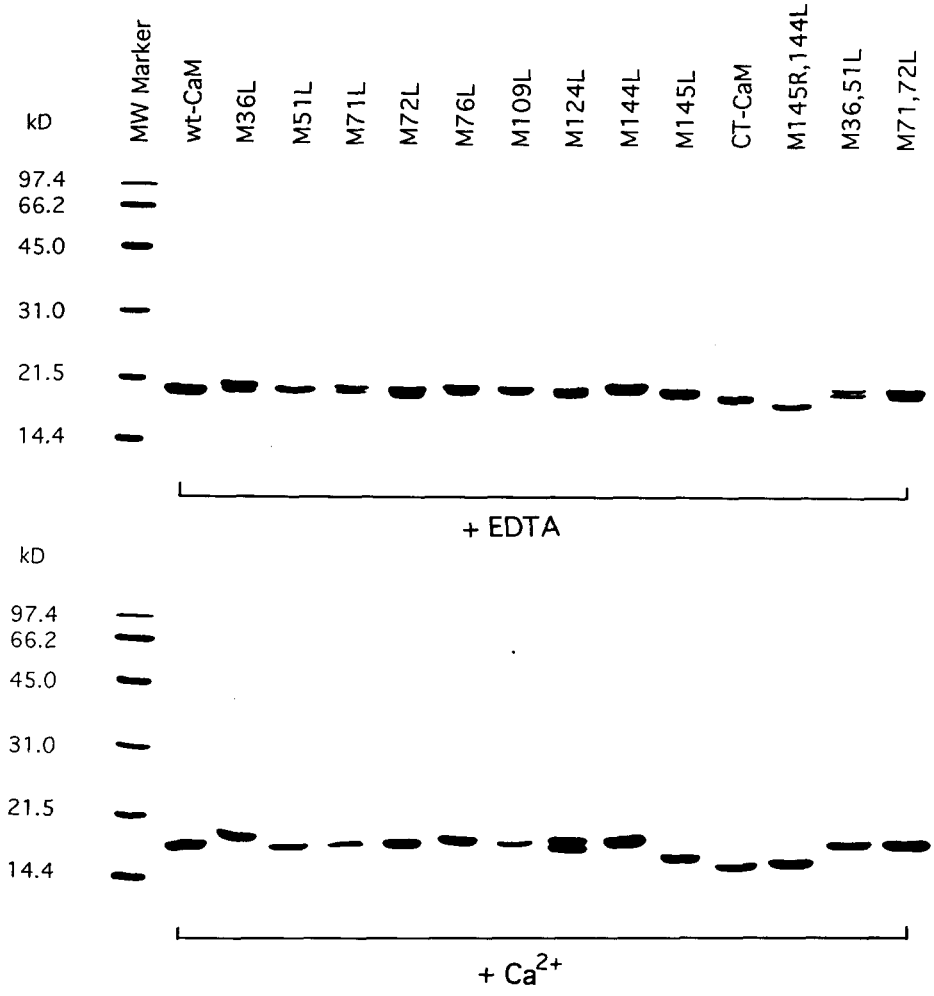

FIG. 1. **SDS-polyacrylamide gel electrophoresis of CaM mutants.** The proteins were run on 15% SDS-PAGE in the presence of 5 mM EDTA (+ *EDTA*) or 5 mM Ca^{2+} (+ Ca^{2+}).

relation with their PDE activation profiles (see below). CT-CaM has a similar Ca^{2+}-dependent mobility shift change compared with wt-CaM, albeit that CT-CaM moves faster both in the presence of EDTA and Ca^{2+}. The introduction of a positively charged Arg residue in position 145 causes the protein to move faster in both conditions. The altered mobility of the M36L-, CT-, and M144R,145L-CaM on SDS-PAGE compared with that of wt-CaM correlated with a lower affinity of these mutants for PDE (see below).

Phosphodiesterase Activation—The functional effects of changing Met residues to Leu residues were studied in detail by measuring the ability of the mutant CaMs to activate the enzyme PDE. Fig. 2*A* shows the activation of PDE by the C-terminal single mutants in comparison with wild-type CaM. All of the C-terminal single Met → Leu mutants can fully activate PDE, and these single mutants have the same K_{act} values (the amount required for 50% maximal activation) as wt-CaM within experimental error. However, the single-point Met → Leu mutations in the N-terminal domain have a more marked effect on the PDE activation profiles (Fig. 2*B*). Most noteworthy is that replacing Met-36 with Leu dramatically decreases the ability of CaM to activate PDE. Other single-point mutations such as M51L-, M71L- and M72L-CaM also lead to a higher K_{act}, although these mutant CaMs can still fully activate PDE. Surprisingly, the M36,51L-CaM can activate PDE better than the single M36L-CaM mutant (Fig. 3). Similar to M36,51L-CaM, the double mutant, M71,72L, is a better activator of PDE than the single mutants M71L and M72L. As expected, the mutation of Met-76 to Leu in the central linker region of CaM does not change the ability of the protein to activate PDE (Fig. 2*B*).

The ability of the C-terminal Met → Leu quadruple mutant (CT-CaM) to activate PDE is shown in Fig. 4. Unlike the C-terminal single-point mutants, CT-CaM has a significantly higher K_{act} than wild-type CaM (5 times higher than wt-CaM), indicating that changing all Met residues in the C-terminal domain decreases the affinity of CaM for PDE significantly. However, CT-CaM can still fully activate PDE, albeit that a much higher concentration of CT-CaM is required to accomplish the full activation.

With a degenerate primer, we have also obtained a M144R, M145L mutant protein. Fig. 5 shows the PDE activation profile for this mutant. The introduction of a single charged residue (Arg) into the C-terminal hydrophobic region virtually abolishes the ability of the protein to activate PDE; it should be noted that M145L-CaM has identical PDE activation properties to wild-type CaM. This result clearly demonstrates the importance of the integrity of both hydrophobic regions in CaM for the activation of a typical target enzyme.

Structural Characterization of Some of the CaM Mutants by NMR—NMR studies were carried out in order to correlate the altered PDE activation profiles for CT-CaM, M36L, and M144R,145L CaM mutants with their structures. Fig. 6*B* shows the (^1H,^{13}C)-HMQC spectrum of Met methyl-^{13}C selectively labeled CT-CaM at 35 °C; and, as expected, only five resonances were detected. These represent the four Met residues in the N-terminal domain and Met-76 in the central linker region of the protein. These five peaks in the spectrum are

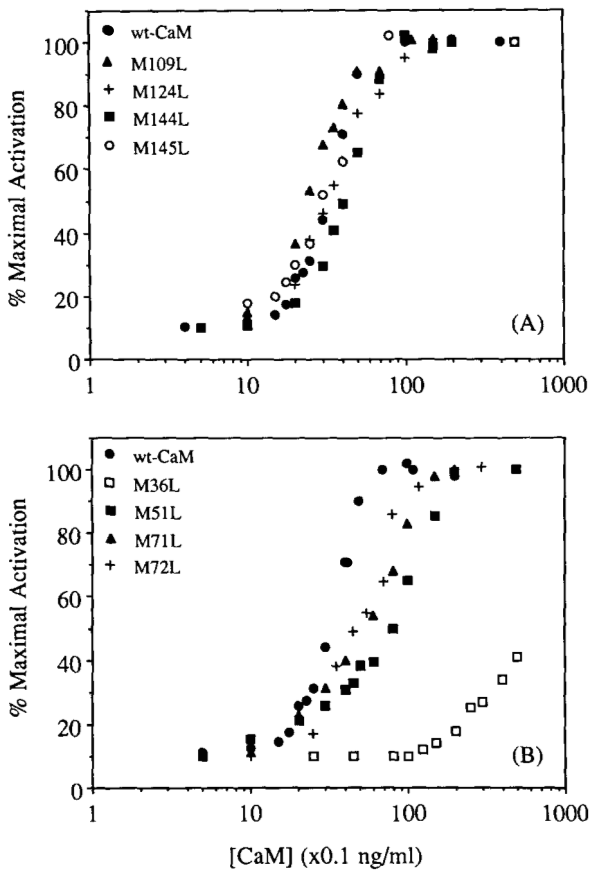

FIG. 2. **Phosphodiesterase activation profiles of the single Met → Leu mutations of CaM.** *A*, C-terminal single point mutations; *B*, N-terminal single point mutations.

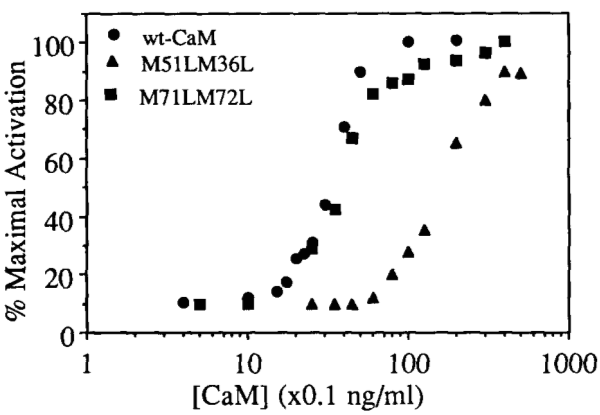

FIG. 3. **Phosphodiesterase activation profiles of the two N-terminal double mutations.**

superimposable with their respective resonances in the (^{1}H,^{13}C)-HMQC spectrum of Met methyl-^{13}C selectively labeled wt-CaM (Fig. 6*A*) acquired under the same conditions. The above result indicates that the mutation of the four Met residues in the C-terminal domain does not have observable structural effects on the N-terminal domain and the central linker region of the protein. The structure of CT-CaM was further characterized by ^{1}H NMR. Fig. 7, *A* and *B*, shows the aromatic and downfield shifted α-proton regions of the Ca^{2+}-form of wt-CaM and CT-CaM. The downfield shifted α-protons originate from the two small antiparallel β-sheets regions of the protein, which are formed by a pair of Ca^{2+}-binding loops in each domain. This region of the proton NMR spectrum is very sensitive to changes in the Ca^{2+} binding and the structure of the protein.

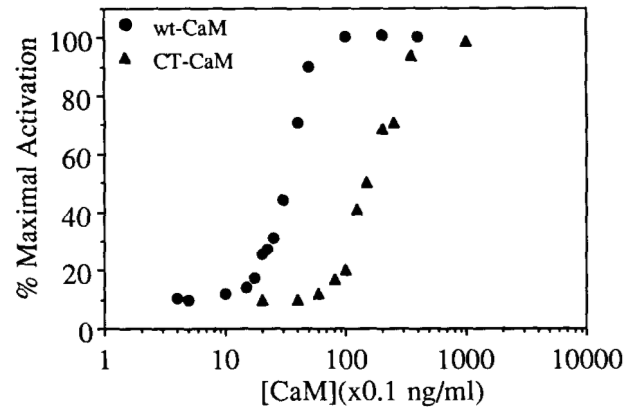

FIG. 4. **Comparison of the phosphodiesterase activation by wt-CaM and CT-CaM.**

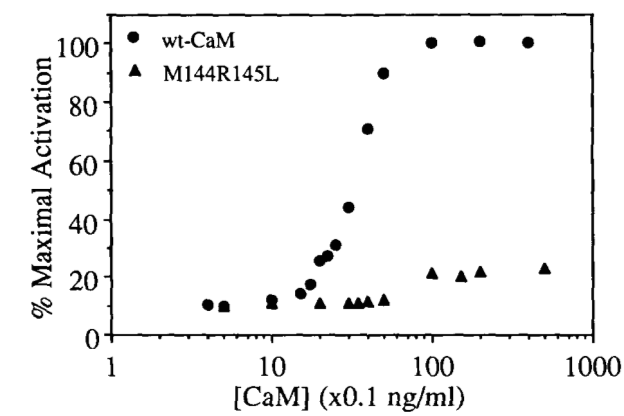

FIG. 5. **Phosphodiesterase activation curves of wt-CaM and M144L,M145R-CaM.**

It is clear from the figure that the downfield shifted α-proton region spectrum of wt-CaM and CT-CaM are virtually the same, and that the aromatic regions of the two proteins are also very similar. The above data strongly suggest that the CT-CaM protein retains an almost identical three-dimensional structure as wt-CaM, even though a total of four Met residues in the C-terminal domain have been replaced with Leu.

The ^{1}H NMR spectrum of M36L-CaM (Fig. 7*C*) is also similar to that of wt-CaM. It indicates that this mutant also maintains a well defined secondary and tertiary structure. However, there are some perturbations in the NMR spectrum; for example, a comparison of the aromatic regions of the ^{1}H NMR spectra of M36L-CaM and wt-CaM shows that the resonance of Phe-68 at 6.8 ppm moves downfield when Met-36 is changed to Leu. Surprisingly, Tyr-138 in the C-terminal domain also experiences some chemical shift changes, which may indicate that the two domains of CaM are communicating with each other in solution. The two small β-sheet regions in the protein appear to be retained, however, as judged from the α-proton peaks around 5.2 ppm. The above preliminary NMR data indicate that mutation of Met-36 to Leu induces some subtle structural changes, and these structural changes may cause the reduced ability of CaM to activate PDE. A complete structure determination by three-dimensional NMR or x-ray crystallography of the M36L-CaM mutant would be necessary to correlate in detail these subtle changes with the dramatic functional effect.

Fig. 7*D* shows the ^{1}H NMR spectrum of the M144R,145L-CaM mutant. Similar to that of CT-CaM, the downfield shifted α-proton region in the ^{1}H NMR spectrum of the mutant is almost identical to that of wt-CaM, and the aromatic region spectra of the mutant and wt-CaM are also very similar, indi-

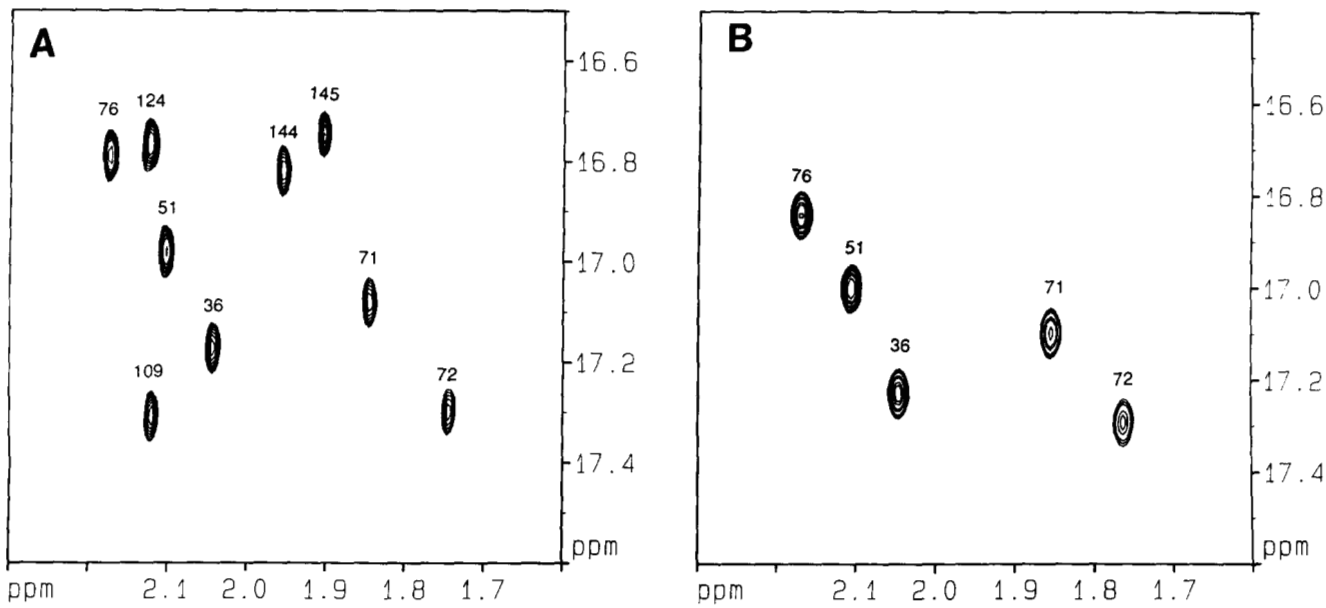

FIG. 6. **(^{1}H,^{13}C)-HMQC spectra of Met-methyl-^{13}C selectively labeled wt-CaM (*A*) and CT-CaM (*B*).** The proteins were saturated with Ca^{2+}, and the pH value of the samples is 7.5. The spectra were acquired at 35 °C. The assignment of the Met residues were made by comparing HMQC spectra of all individual Met → Leu mutants, which were isotopically labeled with 13C-methyl-labeled Met.

cating that the introduction of a single positively charged Arg to the C-terminal hydrophobic patch does not change the structure of the protein. This outcome is perhaps not surprising, because the replacement of an amino acid residue on the surface of a protein with a hydrophilic residue generally should not change the structure of the protein.

Interaction of the CaM-binding Domain of PDE with CaM—As has been shown previously, the Met residues in CaM interact directly with the CaM-binding domains in myosin light chain kinase (Ikura *et al.*, 1992; Meador *et al.*, 1992), CaM-dependent protein kinase IIα (Meador *et al.*, 1993), caldesmon (Zhang and Vogel, 1994b), and the constitutive nitric oxide synthase (Zhang and Vogel, 1994a).[2] Here, we have also studied the involvement of the Met residues of CaM in the formation of the CaM/PDE peptide complex. Fig. 8*B* shows the (^{1}H,^{13}C)-HMQC spectrum of Met-methyl-^{13}C selectively labeled CaM complexed with the PDE peptide. It is obvious from comparing Fig. 8, *B* with *A*, that the formation of the CaM/PDE complex induces significant changes in the chemical shift values as well as the line widths of the Met resonances in CaM. Similar chemical shift changes have also been observed in the complexes of CaM with the CaM binding domains of myosin light chain kinase and constitutive nitric oxide synthase. The results in Fig. 8 suggest that the Met residues in CaM are directly involved in the interaction with the PDE peptide. However, unlike the complexes of CaM with the CaM-binding domains in myosin light chain kinase and constitutive nitric oxide synthase, we have detected a second set of resonances with lower intensity than the nine resonances labeled 1–9 in Fig. 8*B*, suggesting that the interaction between CaM and the PDE peptide may not give rise to one unique complex. Because of this heterogeneity, we have not pursued these studies of the complex any further.

DISCUSSION

In this work, we have used site-directed mutagenesis and enzymatic assays to investigate the roles of the Met residues in CaM. Various studies have suggested that these residues are uniquely suited for a multitarget protein such as CaM (O'Neil and DeGrado, 1990a; Gellman, 1991; Zhang and Vogel, 1994c). The amino acid Leu was chosen to replace the individual Met residues in CaM based on the rationale that Leu is a hydrophobic residue with a slightly shorter side chain compared with Met; moreover, Leu and Met display a similar preference for being in α-helical structure (Lyu *et al.*, 1990; O'Neil and DeGrado, 1990b), and, thus, it is unlikely that it would perturb the secondary structure. We consider Ile a poorer substituent for Met, because it has a preference for β-sheets (Chou and Fasman, 1974; Levitt, 1978; Kim and Berg, 1993). Thus, a Met → Leu mutation is expected to be a relatively conservative substitution in α-helical proteins such as CaM. However, the flexibility of the Leu side chain is significantly lower than that of Met, and the polarizability of Leu is also much lower than that of Met (Gellman, 1991); hence, the Met → Leu mutations can serve as a means of investigating the importance of the flexibility and polarizability of the Met residues in CaM when the protein interacts with its target enzymes such as PDE. NMR studies of the complex of CaM with a peptide encompassing the CaM-binding domain of PDE (Fig. 8) suggested that, similar to what is observed for the myosin light chain kinase and constitutive nitric oxide synthase peptides, all the eight Met residues in the two hydrophobic patches of CaM are involved in the binding of the PDE peptide, while M76 on the central linker region remains unchanged. Consistent with these NMR data, the M76L mutant behaves identical to wt-CaM (Fig. 2*B*). The single mutation of a Met residue in the C-terminal domain of CaM does not have a significant effect on the protein's ability to activate PDE. Likewise, the structure of these mutants seems identical to that of wt-CaM as judged by their ability to interact normally with phenyl-Sepharose and to display a calcium-dependent band shift during SDS-PAGE, as well as from their one-dimensional ^{1}H NMR spectra (data not shown). This outcome may not be unexpected, because the mutated CaM still has three flexible Met residues in the hydrophobic patch allowing for the adjustment of the hydrophobic surface, which remains intact after the introduction of a conservative Met → Leu mutation. However, when all four Met residues in the C-terminal hydrophobic patch are replaced by four Leu residues, the mutated CT-CaM has a significantly lower binding affinity for PDE compared with wt-CaM, al-

[2] M. Zhang, K. Siivari, and H. J. Vogel, unpublished results.

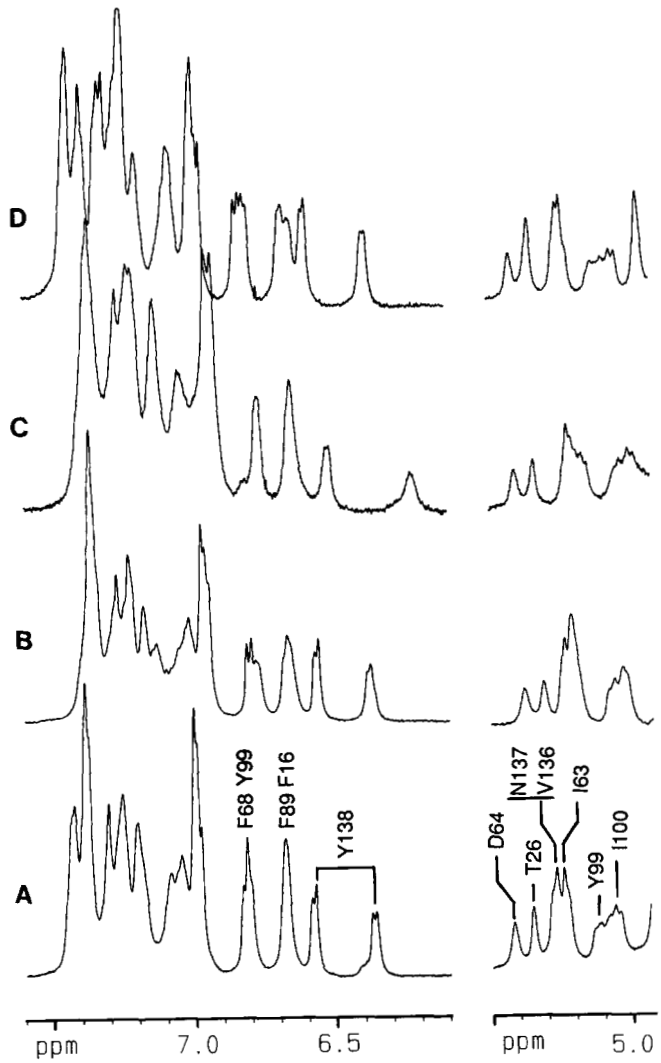

FIG. 7. **The aromatic and downfield shifted α-proton regions of the Ca^{2+}-saturated ^1H NMR spectra of wt-CaM (*A*), CT-CaM (*B*), M36L-CaM (*C*), and M144R145L-CaM (*D*) recorded at 35 °C, pH 7.5.** The assignment labeled in the figure was taken from published results (Ikura *et al.*, 1983, 1985).

though CT-CaM can still fully activate PDE provided a higher concentration of CT-CaM is used to saturate the enzyme (Fig. 4). Our NMR studies of CT-CaM have shown that the mutation of the four Met residues in the C-terminal domain to four Leu residues does not cause serious changes in the structure of the protein (Figs. 6 and 7); hence, a decreased affinity of CT-CaM for PDE may indicate that a hydrophobic surface made of rigid hydrophobic amino acid side chains cannot adapt to provide the optimal structural arrangement to accommodate PDE, compared with a surface that contains several flexible apolar Met residues. At the same time, a hydrophobic patch made of aliphatic residues is predicted to be less sticky than one containing several highly polarizable Met residues (Gellman, 1991).

In contrast to the mutations in the C-terminal domain, all the single mutations in the N-terminal domain have a more significant effect on CaM's PDE activation profile. Each single point mutation decreases CaM's affinity for PDE 1~2-fold. The most striking result is that the M36L mutant is a very poor PDE activator compared with CaM. This may correlate with the fact that Met-36 has the lowest solvent accessibility of all the Met residues in Ca^{2+}-CaM (Babu *et al.*, 1988); changes of buried amino acids may have a larger effect on the structure of the protein. The ^1H NMR spectrum of the M36L mutant has shown that the replacement of Met-36 with Leu indeed induces some subtle structural changes to the protein (Fig. 6), although the protein still keeps a similar overall fold; this structural perturbation may disrupt the hydrophobic surface in the N-terminal domain. Furthermore, all the mutations of the Met residues in the N-terminal domain induce some changes in the NMR spectra of the protein,[2] suggesting that the structure of the N-terminal domain CaM is much more sensitive to these mutations than the C-terminal domain. Previous studies have shown that the first Ca^{2+}-binding domain is absolutely needed for CaM to activate PDE (George *et al.*, 1990). Using chimeric calmodulin-troponin C proteins, George and co-workers (1990) have shown that the replacement of the first Ca^{2+}-binding domain of CaM by that of troponin C results in a protein with an identical PDE activation profile. The amino acid sequences of the first Ca^{2+}-binding domains of CaM and troponin C differ in various positions. However, it seems that the key residues that are required in order to make the protein an efficient PDE activator are present in both proteins; M36 is one of those amino acid residues. Surprisingly, the M36,51L double mutant can activate PDE more efficiently than the single M36L mutant; presumably the introduction of a Leu at position 51 compensates for the structural perturbation that results from the Met-36 → Leu mutation. Indeed, the ^1H NMR spectrum of the M36,51L mutant is more like that of wt-CaM than the M36L mutant (spectrum not shown). Similar results have been obtained with the M71,72L double mutant, which activates better than the single M71L or M72L mutants. The lack of detailed structural information for these mutants makes it difficult to provide a direct explanation for these experimental data at this time. The failure to obtain a quadruple mutant for the N-terminal domain and the larger perturbation on the PDE activation of the single mutations clearly indicates that the requirement for the Met residues in the N-terminal domain is more stringent than in the C-terminal domain.

The introduction of a single positively charged residue (Arg) into the C-terminal hydrophobic patch of CaM (M144R) does not have appreciable structural effects on the protein as indicated by ^1H NMR (Fig. 7). However, this mutant CaM is almost completely inactive in activating PDE. The energetic cost of burying a positive charge in a hydrophobic surface is considerable; obviously, this prevents the proper binding of CaM to PDE. This result highlights the importance of both hydrophobic regions of CaM for the activation of target enzymes such as PDE, as it reinforces the notion that both hydrophobic domains of the protein are an absolute requirement for the proper functioning of CaM. We have attempted to introduce a positive charge into the N-terminal hydrophobic region by mutating Met-72 → Arg. However, the expression of the M72R mutant protein was unsuccessful; thus, at this point no comparison can be made of the importance of keeping the hydrophobic regions intact for both domains of the protein. However, the latter result is again consistent with the greater sensitivity of the N-terminal domain to the mutation of Met residues as discussed above.

The Met residues of CaM are generally well preserved throughout evolution. For example, in the amino acid sequences of CaMs purified from widely divergent organisms such as *Tetrahymena* and *Dicytostelium*, which have over 75% identity with bovine CaM, all Met have been preserved. The yeast CaM purified from *Saccharomyces cerevisiae* has only ≈60% identity with bovine CaM, and, in this protein, only three of the nine Met residues have been retained (Met-36, Met-72, and Met-124). The other six Met residues (at positions 51, 71, 76, 109, 144, and 145) have been replaced by Leu (Davis *et al.*, 1986). Interestingly, this specific yeast CaM is a poor activator of PDE, myosin light chain kinase, and phosphorylase kinase,

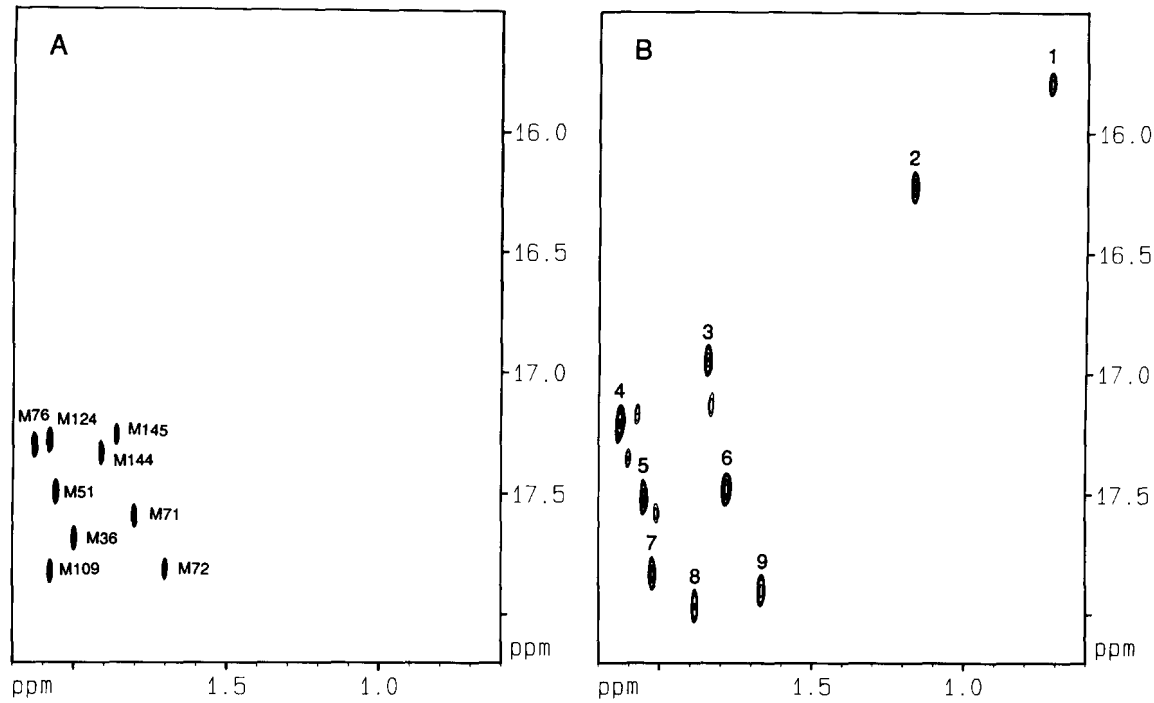

FIG. 8. **(^{1}H,^{13}C)-HMQC spectra of Met-methyl-^{13}C selectively labeled wt-CaM (*A*) and its complex with the PDE peptide at 1:1 ratio (*B*).** The assignment of the Met residues in wt-CaM was obtained by comparing the (^{1}H,^{13}C)-HMQC spectrum of the individual Met-methyl-^{13}C selectively labeled Met → Leu CaM mutant with that of wt-CaM, and this assignment is indicated by the residue number in *A*. The nine resonances in *B* labeled with *numbers* represent nine Met residues from CaM in the complex; no assignment was made in this case. The concentration of the protein in both samples is ≈1 mM, and the spectra were acquired at 25 °C, pH 7.0.

with much higher protein concentrations required to achieve full or partial activation (Luan *et al.*, 1987; Ohya *et al.*, 1987; Davis and Thorner, 1989) (for a summary, see Lu and Means (1993)). It has also been shown that while bovine CaM and yeast CaM bind the same proteins in a yeast extract, the yeast CaM does not bind to many of the proteins that are recognized by the mammalian CaM (Ye and Bretscher, 1992). The structure of this yeast CaM is similar to that of vertebrate CaM (Starovasnik *et al.*, 1993), and, hence, there must be other features of yeast CaM that make it a relatively poor activator. Our data with Met → Leu mutations of mammalian CaM show that such substitutions decrease CaM's affinity for a target protein, which suggests that the same may apply in the case of yeast CaM. Thus, the results obtained with yeast CaM are in general agreement with the conclusion of our study, that substitutions of Met → Leu give rise to a protein that is a poorer activator of target enzymes.

REFERENCES

Babu, Y. S., Bugg, C. E., and Cook, W. J. (1988) *J. Mol. Biol.* **204,** 191–204

Bax, A., Griffey, R. H., and Hawkins, B. L. (1983) *J. Magn. Reson.* **55,** 301–315

Bernstein, H. D., Poritz, M. A., Strub, K., Huben, P. J., Brenner, S., and Walter, P. (1989) *Nature* **340,** 482–486

Charbonneau, H., Kumar, S., Novack, J., Blumenthal, D. K., Griffin, P. R., Shabanowitz, J., Hunt, D., Beavo, J. A., and Walsh, K. A. (1991) *Biochemistry* **30,** 7931–7940

Chattopadhyaya, R., Meador, W. E., Means, A. R., and Quiocho, F. A. (1992) *J. Mol. Biol.* **228,** 1177–1192

Chou, P. Y., and Fasman, G. D. (1974) *Biochemistry* **13,** 211–222

Davis, T. N., and Thorner, J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 7909–7913

Davis, T. N., Urdea, M. S., Masiarz, F. R., and Thorner, J. (1986) *Cell* **47,** 423–431

Eftink, M. R., Andy, M. L., Bystorm, K., Perlmutter, H. D., and Kristol, P. S. (1989) *J. Am. Chem. Soc.* **111,** 6765–6772

Fersht, A. (1985) *Enzyme Structure and Mechanism*, pp. 294–296, W. H. Freeman Press, New York

Gellman, S. H. (1991) *Biochemistry* **30,** 6633–6636

George, S. E., VanBerkum, M. F. A., Ono, T., Cook, R., Hanley, R. M., Putkey, J. A., and Means, A. R. (1990) *J. Biol. Chem.* **265,** 9228–9235

Hiraoki, T., and Vogel, H. J. (1987) *J. Cardiovasc. Pharmacol.* **10,** S14–S31

Klee, C. B., Crough, T. H., and Krinks, M. H. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 6270–6273

Ikura, M., Hiraoki, T., Hikichi, K., Mikuni, T. Yazawa, M., and Yagi, K. (1983) *Biochemistry* **22,** 2568–2572

Ikura, M., Minowa, O., and Hikichi, K. (1985) *Biochemistry* **24,** 4264–4269

Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., and Bax, A. (1992) *Science* **256,** 632–638

Janin, J., Wodak, S., Levitt, M., and Maigret, B. (1978) *J. Mol. Biol.* **125,** 357–386

Kim, C. A., and Berg, J. M. (1993) *Nature* **362,** 267–270

Laemmli, U. K. (1970) *Nature* **227,** 680–685

Levitt, M. (1978) *Biochemistry* **17,** 4277–4285

Lu, K. P., and Means, A. R. (1993) *Endocr. Rev.* **14,** 40–58

Luan, Y., Matsuura, I., Yazawa, M., Nakamura, T., and Yagi, K. (1987) *J. Biochem.* **102,** 1531–1537

Lyu, P. C., Liff, M. I., Marky, L. A., and Kallenbach, N. R. (1990) *Science* **250,** 669–673

Marion, D., and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.* **113,** 967–974

Meador, W. E., Means, A. R., and Quiocho, F. (1992) *Science* **257,** 1251–1254

Meador, W. E., Means, A. R., and Quiocho, F. (1993) *Science* **262,** 1718–1721

Means, A. R., VanBerkum, M. F. A., Bagchi, I., Lu, K. P., and Rasmussen, C. D. (1991) *Pharmacol. & Ther.* **50,** 255–270

Ohya, Y., Uno, I. Ishikawa, T., and Anraku, Y. (1987) *Eur. J. Biochem.* **168,** 13–19

O'Neil, K. T., and DeGrado, W. F. (1990a) *Trends Biochem. Sci.* **15,** 59–64

O'Neil, K. T., and DeGrado, W. F. (1990b) *Science* **250,** 646–651

Putkey, J. A., Slaughter, G. R., and Means, A. R. (1985) *J. Biol. Chem.* **260,** 4704–4712

Rao, S. T., Wu, S., Satyshur, K. A., Ling, K. Y., Kung, C., and Sundaralingam, M. (1993) *Protein Sci.* **2,** 436–447

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sharma, R. K., and Wang, J. H. (1979) *Adv. Cyclic Nucleotide Res.* **10,** 187–198

Sharma, R. K., Taylor, W. A., and Wang, J. H. (1983) *Mehtods Enzymol.* **102,** 210–219

Starovasnik, M., Davis, T. N., and Klevit, R. E. (1993) *Biochemistry* **32,** 3261–3270

Vogel, H. J., Lindahl, L., and Thulin, E. (1983) *FEBS Lett.* **157,** 241–246

Waltersson, Y., Linse, S., Brodin, P., and Grundström, T. (1993) *Biochemistry* **32,** 7866–7871

Ye, R. R., and Bretscher, A. (1992) *Eur. J. Biochem.* **204,** 713–723

Zhang, M., and Vogel, H. J. (1993) *J. Biol. Chem.* **268,** 22420–22428

Zhang, M., and Vogel, H. J. (1994a) *J. Biol. Chem.* **269,** 981–985

Zhang, M., and Vogel, H. J. (1994b) *Biochemistry* **33,** 1163–1171

Zhang, M., and Vogel, H. J. (1994c) *J. Mol. Biol.*, in press

Zhang, M., Yuan, T., and Vogel, H. J. (1993) *Protein Sci.* **2,** 1931–1937

Zhang, M., Huque, E., and Vogel, H. J. (1994) *J. Biol. Chem.* **269,** 5099–5105