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Interaction of Calmodulin with Its Binding Domain of Rat Cerebellar Nitric Oxide Synthase

A MULTINUCLEAR NMR STUDY*

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The intercellular messenger nitric oxide is produced through the action of nitric oxide synthases, a class of enzymes that is regulated by calcium-calmodulin (CaM). In this work, the interaction of CaM with a 23-amino-acid residue synthetic peptide, encompassing the CaM-binding domain of constitutive rat cerebellar nitric oxide synthase (cNOS), was investigated by various NMR methods. Cadmium-113 NMR studies showed that binding of the cNOS peptide increased the affinity of CaM for metal ions and induced interdomain cooperativity in metal ion binding as earlier observed for complexes of CaM with myosin light chain kinase (MLCK) peptides. By using specific isotopically labeled [^{13}C]methyl-Met and selenomethionine-substituted CaM in two-dimensional proton-detected ^{13}C and ^{77}Se NMR studies, we obtained evidence for the involvement of the Met residues of CaM in the binding of the cNOS peptide. These residues form two hydrophobic surface areas on CaM, and they are also involved in the binding of other target proteins. A nitroxide spin-labeled version of the cNOS peptide caused broadening only for NMR resonances in the N-terminal half of CaM, showing that the peptide binds with a C to N orientation to the N- and C-terminal domains of CaM. pH titration experiments of CaM dimethylated with [^{13}C]formaldehyde show that Lys-75 (and Lys-148) experience a large increase in pK_a upon peptide binding; this indicates an unraveling of part of the helical linker region of CaM upon cNOS peptide binding. Taken together, our data show that the cNOS and MLCK peptides bind in a closely analogous fashion to CaM.

The ubiquitous Ca^{2+}-binding protein, calmodulin (CaM),[1] is a small acidic protein of 148 amino acid residues. The protein can regulate many different cellular events by interacting with almost 30 different target enzymes in a Ca^{2+}-dependent manner (for a review, see Means *et al.*, 1991; Vogel, 1994). The CaM-binding domains of the CaM targets are usually contained in a continuous stretch of about 20 amino acid residues. A large number of CaM-binding domains of various CaM targets have a tendency to form basic, amphiphilic α-helices, otherwise these peptides display very low amino acid sequence homology (O'Neil and DeGrado, 1990; James *et al.*, 1995; Vogel and Zhang, 1995). The binding of two Ca^{2+} ions to each domain of CaM induces significant conformational changes and exposes two Met-rich hydrophobic surfaces (Hiraoki and Vogel, 1987; Babu *et al.*, 1988). The interaction of CaM with peptides encompassing the CaM-binding domains of various target enzymes occurs mainly via van der Waals interactions between these two hydrophobic surfaces and the hydrophobic face of the CaM-binding domain of its targets (Ikura *et al.*, 1992; Meador *et al.*, 1992, 1993; Zhang and Vogel, 1994b). The Met residues in the hydrophobic surfaces play important roles by allowing CaM to bind to many targets in a sequence-independent manner (O'Neil and DeGrado, 1990; Vogel *et al.*, 1990; Gellman, 1991; Vogel, 1994; Zhang and Vogel, 1994c; Zhang *et al.*, 1994a). The crystal structure of calcium-CaM reveals a dumbbell-shaped molecule, where the two globular domains of the protein are connected by a long, solvent-exposed α-helix (Babu *et al.*, 1988). This central linker region is very flexible in solution (Ikura *et al.*, 1991; Spera *et al.*, 1991; Barbato *et al.*, 1992), and its plasticity is one of the main reasons why CaM can interact effectively with such a wide spectrum of target proteins (Ikura *et al.*, 1992; Meador *et al.*, 1992, 1993; Zhang and Vogel, 1994b). In complexes of CaM with its binding domains of MLCKs (Ikura *et al.*, 1992; Roth *et al.*, 1992; Meador *et al.*, 1992) and CaM-dependent kinase II (Meador *et al.*, 1993), the two globular domains of the protein essentially retain the same conformation, but in all cases the central helix of CaM unravels in a specific manner, allowing CaM to accommodate α-helical amphiphilic CaM-binding domains of different lengths.

Nitric oxide is a small gaseous messenger molecule which can regulate diverse physiological processes, such as smooth muscle relaxation, platelet inhibition, neurotransmission, immune regulation, and blood vessel dilation (for reviews, see Nathan, 1992; Feldman *et al.*, 1993; Bredt and Snyder, 1994; Nathan and Xie, 1994). The cellular level of nitric oxide is regulated by the action of nitric oxide synthases (NOS). There are two distinct isoforms of NOS, a constitutively expressed brain and endothelial NOS (Bredt and Snyder, 1990; Bredt *et al.*, 1991; Pollack *et al.*, 1991; Sessa *et al.*, 1992) and a cytokine-induced isoform from macrophages (Stuehr *et al.*, 1991; Xie *et al.*, 1992). The constitutively expressed NOS (cNOS) are activated by CaM in a Ca^{2+}-dependent manner; the inducible NOS are not further activated by exogenous Ca^{2+}-CaM, since they contain CaM as an integral, tightly bound subunit (Nathan and

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[1] The abbreviations used are: CaM, calmodulin; 1D, one-dimensional; 2D, two-dimensional; cNOS, constitutive nitric oxide synthase; FTIR, Fourier transform infrared spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond correlation; MLCK, myosin light chain kinase; T_1, spin-lattice relaxation time; T_2, spin-spin relaxation time.

Xie, 1994). Synthetic peptides encompassing the putative CaM-binding domain of rat brain cNOS (Bredt *et al.*, 1991) have been shown to bind to CaM in a Ca^{2+}-dependent manner with a 1:1 stoichiometry and a K_D of ≈2 nM (Vorherr *et al.*, 1993; Zhang and Vogel, 1994a). From CD and isotope-edited FTIR studies, it is known that the cNOS peptide binds to CaM in an α-helical conformation (Zhang and Vogel, 1994a; Zhang *et al.*, 1994b). However, further details about the mode of binding and the location of the cNOS peptide binding site on CaM are lacking at present.

In the present work, we have used 1D and 2D ^{1}H, ^{13}C, ^{77}Se, and ^{113}Cd NMR spectroscopy to study the interaction of the cNOS peptide with CaM. The involvement of the functionally important Met residues of CaM for binding the cNOS peptide was studied by ^{1}H,^{13}C HMQC spectroscopy and ^{1}H,^{77}Se HMBC spectroscopy. From the pK_a titration behavior of Lys-75, we have obtained information about the unraveling of the central helix of CaM upon peptide binding (Zhang and Vogel, 1993). Cadmium-113 NMR spectra were acquired to study the effect of peptide binding on the metal-ion binding sites (Vogel and Forsén, 1987; Ikura *et al.*, 1989). Finally, a paramagnetic nitroxide spin-labeled cNOS peptide was prepared to map the orientation of the cNOS peptide in its complex with CaM.

MATERIALS AND METHODS

All chemicals (analytical grade) were obtained commercially. The nitroxide spin labeling reagent 4-(2-bromoacetamido)-2,2,6,6-tetramethylpiperidine-*N*-oxyl was purchased from Sigma. The 23-residue cNOS peptide KRRAIGFKKLAEAVKFSAKLMGQ was obtained and handled as described previously (Zhang and Vogel, 1994a). Preparations of wild-type CaM, CaM mutants, isotopically labeled $^{13}CH_3$-Met-CaM, and natural abundance ^{77}Se-Met-incorporated CaM have been described in detail in our earlier reports (Zhang and Vogel, 1993, 1994b, 1994c; Zhang *et al.*, 1994a). Reductive methylation of the Lys residues in CaM with ^{13}C-labeled formaldehyde was performed according to published procedures; this provides a convenient means of measuring individual pK_a values in CaM (Huque and Vogel, 1993; Zhang and Vogel, 1993). The CaM·peptide complexes used in this study were prepared in essentially the same manner as the complex of CaM with the CaM-binding domain of MLCK (Zhang and Vogel, 1993).

Spin Labeling of the cNOS Peptide—The spin-labeled cNOS peptide in which the single Met residue was specifically reacted was prepared following published procedures (Lundblad and Noyes, 1984; Musci *et al.*, 1988). Briefly, the cNOS peptide was incubated with a 10-fold excess of 4-(2-bromoacetamido)-2,2,6,6-tetramethylpiperidine-*N*-oxyl for 24 h at room temperature in 0.2 M acetate buffer, pH 3.0. Under these conditions this reagent will react specifically with the sulfur atom of the single Met in the peptide (Musci *et al.*, 1988). The labeled cNOS peptide (yield 50%) was separated from the unreacted cNOS peptide by reverse-phase high performance liquid chromatography; only a single peak was observed for the modified peptide indicating that no side reactions have taken place.

NMR Experiments—All ^{1}H, ^{13}C and ^{77}Se NMR spectra were acquired on a Bruker AMX500 NMR spectrometer equipped with a 5-mm broadband inverse detection probe at 25 °C unless otherwise indicated. A total of 128 scans were collected for the 1D ^{1}H NMR spectra with a sweep width of 5000 Hz. ^{1}H,^{13}C HMQC spectra were recorded in the phase-sensitive mode (Bax *et al.*, 1983). The two-dimensional natural abundance ^{1}H,^{77}Se HMBC spectrum of the SeMet-CaM·cNOS peptide complex was recorded in the magnitude mode (Zhang and Vogel, 1994c). All NMR spectra were processed on a Bruker X32 computer using commercially available Bruker UXNMR software.

Cadmium-113 NMR experiments were performed on a Bruker AM400 widebore NMR spectrometer, which was equipped with a 10-mm broadband probe. All spectra were recorded at a frequency of 88.75 MHz. Isotopically enriched ^{113}CdO (94.8%) was purchased from MSD Isotopes. A 100 mM stock solution of $^{113}Cd(ClO_4)_2$ (pH ≈ 1 in D_2O) was used for the preparation and titration of the samples. All ^{113}Cd NMR samples were prepared in 25% (v/v) D_2O, 100 mM KCl, 20 mM Tris, pH 7.5 ± 0.05. For the $^{113}Cd_4^{2+}$-CaM sample titrated with cNOS peptide, microliter quantities of a concentrated peptide solution were added into the sample. The apo-CaM-cNOS peptide mixture was prepared by gradually adding cNOS peptide to a diluted apo-CaM solution until a 1:1 ratio was reached. The mixture was then lyophilized and redissolved in 2.0 ml of buffer. The ^{113}Cd NMR acquisition parameters are as follows: a 45° flip angle, acquisition time 0.14 s, relaxation delay 0.5 or 5 s, sweep width 30 kHz, 8 K data points, and 70,000 scans. The temperature of all experiments was 298 K. The total acquisition time for a 0.5 s delay experiment is 12 h, 40 min. Each FID was zero-filled once and an exponential line broadening of 30 Hz was applied prior to Fourier transformation. All ^{113}Cd spectra are referenced to external 100 mM $^{113}Cd(ClO_4)_2$ in D_2O.

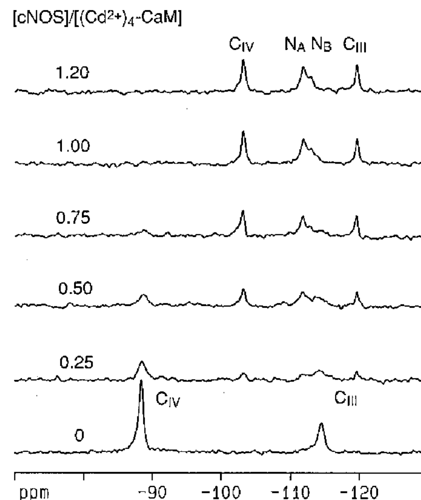

FIG. 1. **Cadmium-113 NMR spectra of Cd^{2+}-saturated CaM (0.59 mM protein concentration, pH 7.5) titrated with cNOS peptide.** These spectra were acquired with a 0.5 s relaxation delay. The ratio of peptide to protein is indicated in the figure.

RESULTS

Binding of the cNOS Peptide Induces Total Cooperativity in Metal Ion Binding—Cadmium-113 NMR titration experiments showed that both domains of CaM are affected simultaneously by the binding of the cNOS peptide. It should be noted that only two signals are detected for $^{113}Cd_4^{2+}$-CaM in the absence of the peptide; these represent the two cations bound in the two C-terminal sites of CaM; those in the N-terminal part are not detected due to an exchange process that broadens both resonances beyond detection (Forsén *et al.*, 1986). Fig. 1 shows the spectra of $^{113}Cd_4^{2+}$-CaM titrated with the cNOS peptide; upon addition of the peptide a total of four resonances is detected, as observed earlier with other target-bound CaMs (see Table I). The spectra reveal that the cNOS peptide binds to $^{113}Cd_4{}^{2+}$-CaM in slow exchange on the ^{113}Cd NMR time scale. All of the four new resonances increase simultaneously in intensity with the concomitant disappearance of the two signals from $^{113}Cd_4^{2+}$-CaM. These four signals can be tentatively assigned to the four Cd^{2+} ions bound to the four calcium-binding sites in the CaM-cNOS peptide complex, by comparison with values obtained for other complexes (see Table I). No minor signals are detectable in the spectra, suggesting that the peptide binds in only one orientation. Upon integration of the peaks, we found that the 1:1 complex only equals to 2 equivalents of Cd^{2+}, as does the $^{113}Cd_4^{2+}$·CaM complex. It is quite common in ^{113}Cd NMR that spectra are not fully relaxed if the relaxation delay used is short (Coleman, 1993). Therefore we acquired spectra with a 5 s delay for both $^{113}Cd_4^{2+}$-CaM and the $^{113}Cd_4^{2+}$·CaM·cNOS peptide complex at the same protein concentrations. The integration data obtained for these samples showed that the protein-peptide complex equals 3.7 equivalents of $^{113}Cd^{2+}$, which is close to the expected 4 equivalents; the integrated intensity of $^{113}Cd_4^{2+}$-CaM remains 2.0 equivalents indicating a significant change in the T_1 relaxation rate upon the binding of the peptide to the protein. Similar integration results have also been obtained for $^{113}Cd_4^{2+}$-CaM titrated with the skeletal mus-

TABLE I
Chemical shifts measured for the protein-bound ^{113}Cd signals in the presence of peptides or drugs

Protein	Chemical shifts (ppm)				Refs.
	C_{IV}	N_A	N_B	C_{III}	
CaM[a]	−88.4	NO[b]	NO[b]	−114.4	Andersson *et al.*, 1983; Thulin *et al.*, 1984
CaM/cNOS	−103.1	−111.8	−112.9	−119.6	This study
CaM/skMLCK	−101.9	−113.3	−117.7	−122.1	Ikura *et al.*, 1989; Ohki *et al.*, 1993
CaM/mastoparan	−102	−111	−112	−121	Linse *et al.*, 1986
CaM/melittin[a]	−96.1	−115.0	−116.4	−118.7	Yuan and Vogel, unpublished results
CaM/trifluoperazine[a]	−99.1	−112.9	−115.2	−118.1	Thulin *et al.*, 1984

[a] C-terminal C_{III}, C_{IV} resonances (calcium binding sites III and IV) and N-terminal N_A, N_B resonances (sites I and II are not distinguished) have been assigned in these studies by using tryptic and thrombic proteolytic fragments of CaM.
[b] NO, not observable due to exchange broadening (see Forsén et al., 1986).

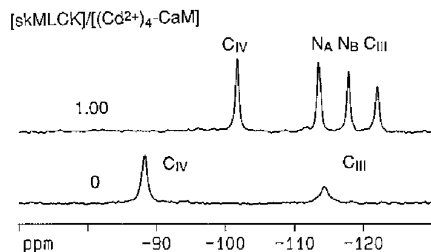

FIG. 2. **Cadmium-113 NMR spectra of Cd^{2+}-saturated CaM (0.53 mM, pH 7.5) and the 1:1 complex with MLCK peptide.** These spectra were acquired with a 5-s relaxation delay, so peak intensity can be compared directly. Note the obvious line width narrowing in the spectra of the complex.

cle MLCK peptide (see Fig. 2).

We also performed a titration of the apo-CaM-cNOS peptide mixture with $^{113}Cd(ClO_4)_2$. Under these conditions, four ^{113}Cd NMR peaks increased simultaneously in intensity until a 4:1 ratio of metal ions to protein was reached (Fig. 3). The chemical shifts obtained in this experiment were consistent with those in Table I. The parallel increase of the four signals shows that CaM possesses total positive cooperativity in metal ion binding in the presence of the cNOS peptide. This is not evident in the absence of the peptide, but it can be induced by the binding of MLCK peptide, as well as other targets (Forsén *et al.*, 1986; Ikura *et al.*, 1989; Vogel, 1994). The line widths of the various ^{113}Cd resonances are listed in Table II.

The cNOS Peptide Binds to the Met-rich Hydrophobic Surfaces of CaM—The two hydrophobic surfaces of CaM that are generally involved in the binding of the CaM-binding domains of target proteins are very rich in Met residues (Babu *et al.*, 1988; O'Neil and DeGrado, 1990). Therefore, we studied the interaction between CaM and the cNOS peptide by heteronuclear NMR spectroscopic techniques using $^{13}CH_3$-Met isotopically labeled CaM (Siivari *et al.*, 1995). Fig. 4, *A* and *B*, show the ^1H,^{13}C HMQC spectra of the methyl groups of the Met residues from Ca^{2+}·CaM and the CaM·cNOS peptide complex. The assignment of the Met resonances were obtained by using individual Met → Leu mutants of the protein (for description see Zhang *et al.*, 1994a; Siivari *et al.*, 1995). With the exception of Met-76 which is part of the central linker of CaM, all the Met residues in the hydrophobic patches undergo significant chemical shift changes upon binding the cNOS peptide (see Table III). Because of the sensitivity of the ^{77}Se chemical shift to changes in local environment (Luthra and Odom, 1986), the effects of complex formation on the Met residues in CaM were also studied by ^{77}Se NMR using CaM with Se-Met incorporated for Met (Zhang and Vogel, 1994c). Again, the binding of the cNOS peptide causes drastic chemical shift changes for the resonances of the 8 Se-Met residues from the two hydrophobic patches (see Table III). These results demonstrate that the two

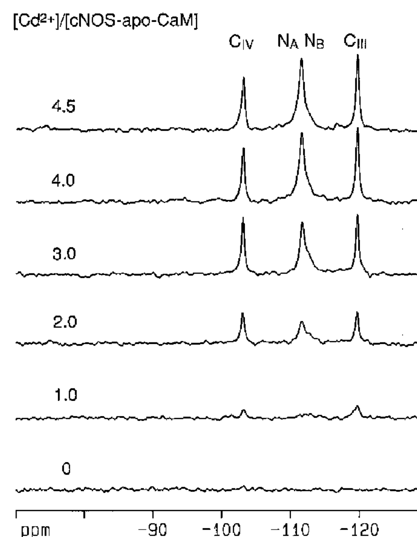

FIG. 3. **Cadmium-113 NMR spectra of a 1:1 mixture of apo-CaM and cNOS peptide titrated with Cd^{2+} (0.82 mM protein concentration, pH 7.5).** The relaxation delay used was 0.5 s. The amount of Cd^{2+} added is indicated in the figure.

TABLE II
Line width of ^{113}Cd resonances of CaM, CaM·cNOS, and CaM·MLCK peptide complexes

Protein	Line width (Hz)				
	Free CD^{2+a}	C_{IV}	N_A	N_B	C_{III}
CaM	68	56	NO[b]	NO[b]	105
CaM·cNOS	53	35	50	68	30
CaM·MLCK	57	16	23	15	26

[a] This signal (around 44 parts/million) is only observed when slightly more than 4 equivalent of Cd^{2+} are added.
[b] NO = not observable due to exchange broadening.

Met-rich hydrophobic regions of CaM are directly involved in the binding of the cNOS peptide.

The Conformation of the Central Helix of Ca^{2+}-CaM Changes upon Binding to the cNOS Peptide—The possibility of conformational changes in the central helix/linker region of CaM upon binding of the cNOS peptide was evaluated by determining the pK_a changes of Lys-75. It has been shown that the unraveling of the helical part of this region upon complex formation (Ikura *et al.*, 1992) is accompanied by a drastic increase in the pK_a of this residue (Zhang and Vogel, 1993). Fig. 5 shows the ^1H,^{13}C HMQC spectra of reductively dimethylated CaM and its K75R and K148Q mutants complexed with the cNOS peptide. From these data, it is immediately apparent that the missing resonances belong to Lys-75 and Lys-148, respectively. pH titration of the ^{13}C-methylated-CaM·cNOS

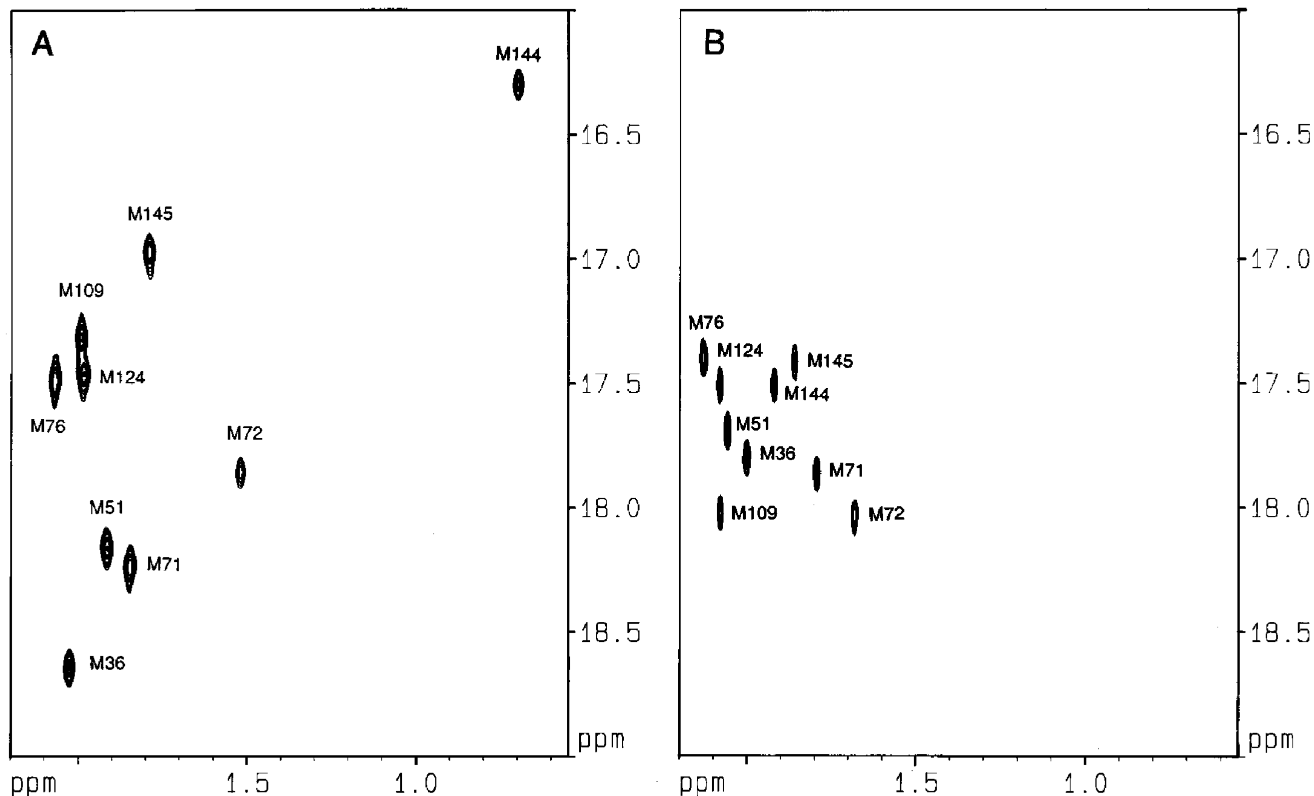

FIG. 4. **$^1H,^{13}C$ HMQC NMR spectra of isotopically labeled [^{13}C]*methyl*-Met calmodulin in the presence (*A*) and absence (*B*) of a saturating amount of the cNOS peptide.** Note the large chemical shift dispersion upon peptide binding (99.9% D_2O, 100 mM KCl, pD 7.5, 25 °C).

TABLE III

^1H, ^{13}C and ^{77}Se chemical shift values of Met or Se-Met residues in Ca^{2+}-CaM, CaM·cNOS and CaM·MLCK peptide complexes

Residue no.	^1H chemical shift (ppm)[a]			^{13}C chemical shift (ppm)[a]			^{77}Se chemical shift (ppm)		
	CaM	CaM·cNOS	CaM·MLCK[b]	CaM	CaM·cNOS	CaM·MLCK[b]	CaM[c]	CaM·cNOS[d]	CaM·MLCK[d,e]
36	2.04	2.03	2.27	17.10	18.64	18.28	70.0	88.6	62.7
51	2.10	1.92	2.04	16.98	18.16	18.20	91.1	104.1	82.9
71	1.83	1.85	0.96	17.16	18.23	16.92	106.8	99.2	111.1
72	1.73	1.52	1.89	17.34	17.86	14.51	51.9	69.5	34.1
76	2.17	2.07	2.09	16.70	17.50	16.78	77.6	73.0	77.4
109	2.12	1.98	1.91	17.33	17.32	17.80	57.0	71.4	83.9
124	2.12	1.97	0.98	16.81	17.47	15.22	97.7	98.5	102.3
144	1.96	0.69	1.66	16.81	16.31	16.51	108.0	110.2	87.8
145	1.90	1.78	1.08	16.71	16.98	18.43	48.7	74.9	40.1

[a] Data were acquired at 25 °C.
[b] Data obtained from Siivari *et al.* (1995).
[c] Data were acquired at 30 °C.
[d] Data were acquired at 55 °C.
[e] Data obtained from Zhang and Vogel (1994c).

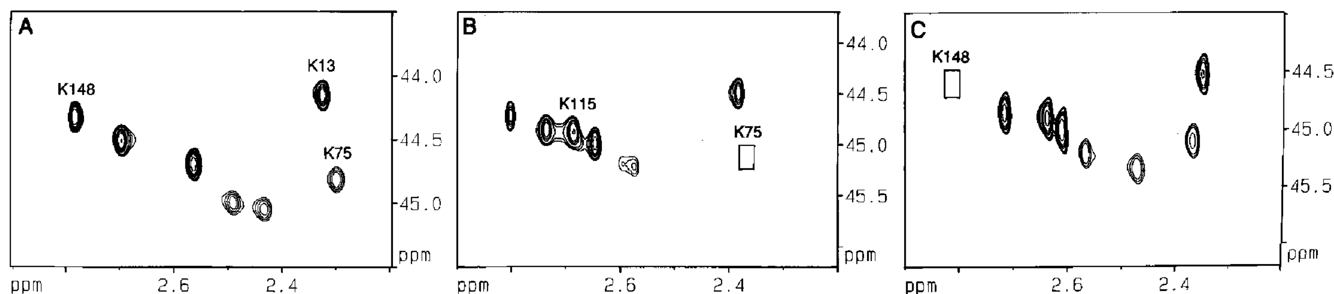

FIG. 5. **$^1H,^{13}C$ HMQC NMR spectra of ^{13}C-dimethylated wild type calmodulin (*A*) and the mutant K75R (*B*) and K148Q (*C*).** The procedure for isotopic dimethylation of CaM was described in detail elsewhere (Huque and Vogel, 1993; Zhang and Vogel, 1993).

peptide complex allowed us to obtain the pK_a values of the individual Lys residues of CaM (see Table IV). Fig. 6 shows the pH titration curves of Lys-75 in Ca^{2+}·CaM and in the CaM·cNOS peptide complex. Lys-75 displays a significant pK_a change from 9.29 in CaM to 9.94 in the complex. Similar to what was observed for the CaM·MLCK peptide complex, Lys-148 also shows a large pK_a change upon complex formation with the cNOS peptide, whereas the other Lys residues only

TABLE IV
pK_a values of the dimethyllysine residues in Ca^{2+}-CaM, CaM·cNOS and CaM·MLCK peptide complexes

Residue no.	CaM[a]	CaM·cNOS	CaM·MLCK[a]
13	10.09	10.24	10.08
21	9.88	10.02	10.12
30	9.84	9.92	10.03
75	9.29	9.94	10.12
77	10.23	10.49	10.21
94	9.65	9.89	9.62
148	10.00	10.90	10.55

[a] Data obtained from Zhang and Vogel (1993).

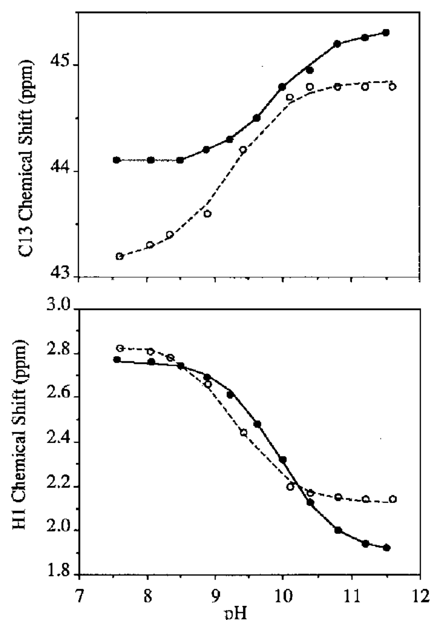

FIG. 6. **pH titration curves determined for the dimethylated Lys-75 resonance of Ca_4^{2+}-CaM (E) and Ca_4^{2+}-CaM-cNOS (●).**

experience minor changes.

The C-terminal Region of the cNOS Peptide Binds to the N-terminal Hydrophobic Surface of CaM—Introduction of nitroxide spin labels covalently attached to ligands provides a useful means of determining the binding orientation of ligands on proteins (see for example de Jong *et al.*, 1988; Penington and Rule, 1992; Folkers *et al.*, 1993; Girvin and Fillingame, 1995). We have utilized a Met nitroxide spin-labeled cNOS peptide in order to map out the orientation of the CaM-bound cNOS peptide with respect to the two domains of the protein. Fig. 7*A* shows the $^{1}H,^{13}C$ HMQC spectrum of the $^{13}CH_3$-Met-labeled CaM complexed with the nitroxide spin-labeled cNOS peptide. Comparing Figs. 7*A* and 4*B*, it is clear that the four Met resonances from the C-terminal domains of CaM do not show any chemical shift and line width changes, whereas the Met resonances from the N-terminal domain undergo significant chemical shift as well as line width changes (the contour levels for the three visible N-terminal resonances were about half of those in the C-terminal domain). Most noticeably, a resonance tentatively assigned to Met-72 was broadened beyond detection by the spin label in the cNOS peptide. The chemical shift changes observed for Met-36, Met-51, and Met-71 result from the conformational perturbation induced by the relatively large hydrophobic spin labeling reagent attached to the cNOS peptide, as these persist following the reduction of the spin label (see below). In order to ascertain that the broadening of the resonances of the N-terminal Met residues in Fig. 7*A* results from the paramagnetic effect of the spin label, we have added ascorbic acid, which can abstract the single electron from the

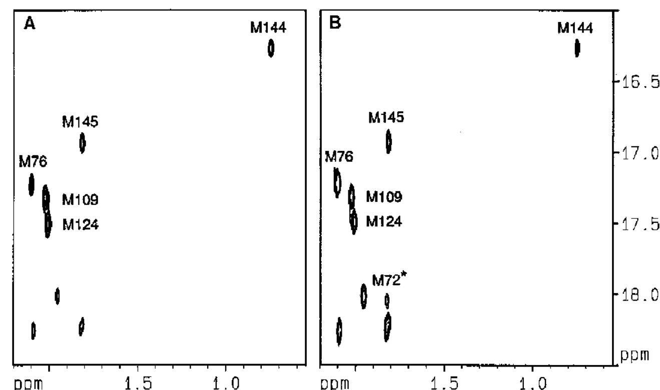

FIG. 7. **$^{1}H,^{13}C$ HMQC NMR spectra of selectively labeled-[^{13}C]-*methyl*-Met calmodulin.** *A*, in the presence of the spin-labeled cNOS peptide; *B*, after addition of a 10-fold excess of ascorbic acid. Note that the resonances for the four C-terminal Met residues have not shifted or broadened (compare with Fig. 4*A*). For further explanation see text.

nitroxide (Kosen *et al.*, 1986). Fig. 7*B* provides the spectrum of the complex recorded 24 h after the addition of ascorbic acid. The reappearance of the Met-72 and the increased intensity for the other N-terminal Met resonances demonstrates that the broadening effect results from the close proximity of the spin label (in *panel* 7*B*, the contour levels for Met-36, Met-51, and Met-71 are identical to those in the C-terminal domain). Hence, the C-terminal end of the cNOS peptide binds to the N-terminal hydrophobic surface of the protein.

DISCUSSION

The synthetic cNOS peptide used in this study has earlier been shown to contain the complete CaM-binding domain of rat brain cNOS (Vorherr *et al.*, 1993; Zhang and Vogel, 1994a). NMR studies of the isolated cNOS peptide have also shown that it can adopt a nascent α-helical structure in aqueous solution that it forms a stable α-helix in aqueous trifluoroethanol (Zhang and Vogel, 1994; Vogel and Zhang, 1995) and that the α-helix has a typical amphiphilic structure as seen in many other CaM-binding peptides (O'Neil and DeGrado, 1990; James *et al.*, 1995). Using CD and isotope-edited FTIR spectroscopy, we have demonstrated that the cNOS peptide also adopts an α-helical structure in its complex with CaM (Zhang *et al.*, 1994b). Here, we have used ^{1}H, ^{13}C, ^{77}Se and ^{113}Cd NMR spectroscopic techniques to further characterize the details of the interaction between the cNOS peptide and CaM. It is clear from our NMR data that the cNOS peptide binds to both domains of calcium-CaM in slow exchange on the NMR time scale(s), since one observes distinct sets of resonances for the complex and free CaM. These data also show that the cNOS peptide forms a stable 1:1 complex with CaM, as the spectral changes in the titrations are completed once the amount of the cNOS peptide reaches this molar ratio.

Cadmium-113 NMR provides a useful method for the study of various metalloproteins including a range of calcium-binding proteins (for reviews see Vogel and Forsén, 1987; Coleman, 1993). For example, the binding of metal ions to CaM, its proteolytic fragments, and its complex with drugs and model-target CaM-binding domain peptides have been studied in this fashion (Andersson *et al.*, 1983; Thulin *et al.*, 1984; Forsén *et al.*, 1986; Linse *et al.*, 1986; Ikura *et al.*, 1989; Ohki *et al.*, 1993). Here we have performed titration studies of the binding of the cNOS peptide to CaM by ^{113}Cd NMR. The four ^{113}Cd resonances in the CaM·cNOS peptide complexes could be tentatively assigned to the four Ca^{2+}-binding sites of CaM, by comparison to earlier studies (see Table I). When the cNOS apo-

CaM mixture was titrated with cadmium, four ^{113}Cd signals simultaneously increased in intensity, which showed that cooperativity in metal ion binding is induced between the N and C domains of CaM in the presence of the peptide. This is likely what will happen *in vivo* because CaM always exists in the presence of CaM-binding proteins in the living cell (Vogel, 1994). Integration of the ^{113}Cd NMR spectra indicated that the ^{113}Cd T_1 relaxation time in the complex is longer than in $^{113}Cd_4^{2+}$-CaM. Also the T_2 relaxation time for the complex appears to be longer than that in Cd_4^{2+}-CaM from the obvious line width narrowing ($\Delta\nu_{1/2}$) in the complex (Table II); however, it is important to realize that chemical exchange processes, rather than T_2 relaxation generally play a determining role for the line width of protein-bound Cd^{2+} ions (Kördel *et al.*, 1992; Aramini *et al.*, 1995). Because chemical shift anisotropy contributes significantly to the ^{113}Cd relaxation of similar protein-bound ions at 9.4 Tesla (Kördel *et al.*, 1992; Aramini *et al.*, 1995), we can calculate the T_2 (and line width) values for Cd_4^{2+}·CaM and the Cd_4^{2+}·CaM·MLCK or cNOS complexes, assuming correlation times of 8.5 and 10 ns, respectively, at 25 °C and using a chemical shift anisotropy value $\Delta\sigma = 91$ parts/million, as determined for the homologous protein calbindin (Kördel *et al.*, 1992). If we assume that the ^{113}Cd relaxation can be attributed completely to chemical shift anisotropy and dipole-dipole relaxation, we can calculate line widths of 0.87 and 1.02 Hz for CaM and CaM·peptide complexes, respectively. These values are obviously smaller than the line widths measured in our NMR spectra, suggesting that some chemical exchange processes are involved. Because the line widths of the free Cd^{2+} signals did not change significantly upon peptide addition (Table II), we do not think that exchange between protein-bound and free Cd^{2+} is involved. A possible explanation is that some fluctuations exist in the Ca^{2+}-binding sites corresponding to slightly different Cd^{2+} chemical shifts (Vogel and Forsén, 1987). Be that as it may, our ^{113}Cd NMR are consistent with the notion that the CaM·cNOS peptide complex, as well as the CaM·MLCK peptide complex, provide a more constrained Ca^{2+}-binding environment than Ca^{2+}-CaM.

The binding of CaM with its target peptides takes place mainly via van der Waals interactions between the Met-rich hydrophobic surfaces of CaM and the hydrophobic face of the CaM-target peptides (Ikura *et al.*, 1992; Meador *et al.*, 1992, 1993; Zhang and Vogel, 1994b; Zhang *et al.*, 1994a). In particular, the polarizability of the Met sulfur atoms can contribute significantly to the binding affinity (Gellman, 1991). Selectively $^{13}CH_3$-Met isotopically labeled and Se-Met containing CaM were used to study the involvement of the Met residues in the interaction with the cNOS peptide. The dramatic chemical shift changes observed for all of the Met and Se-Met residues in the two hydrophobic surfaces of CaM upon complex formation clearly indicate that the cNOS peptide also interacts with CaM through hydrophobic interactions. An analysis of the chemical shift changes of the Met ^{1}H, ^{13}C, and ^{77}Se resonances upon binding of the MLCK and cNOS peptides (see Table III) show that the changes induced by MLCK and cNOS are not identical. This behavior is expected, as the different amino acid sequence of these two target peptides would give rise to distinct bound conformations for the Met side chains of CaM.

The helical parts of the central linker region of CaM unravel further upon complex formation (Ikura *et al.*, 1992; Meador *et al.*, 1992, 1993). It is anticipated that the linker region of CaM will also unravel when CaM forms a complex with the cNOS peptide. We have shown earlier that the binding of a target peptide to the hydrophobic surfaces of CaM will displace the side chain of Lys-75 from the N-terminal hydrophobic surface; this process may induce the unraveling of the central α-helix (Vogel, 1994). This displacement causes a significant increase in its pK_a (Zhang and Vogel, 1993). Here we have observed that the pK_a of Lys-75 changes from 9.29 to 9.94 when CaM forms a complex with the cNOS peptide, and this increase is comparable to what was seen for MLCK (Zhang and Vogel, 1993, see also Table IV). This suggests that the helical parts of the linker region of CaM also undergo a conformational change upon the formation of the complex with the cNOS peptide. The pK_a of Lys-148 also increases significantly when CaM binds to the cNOS peptide, and this again resembles the results with the MLCK peptide (Zhang and Vogel, 1993). By analogy to Lys-75, it is believed to be caused by a displacement of the Lys-148 side chain from an orientation on top of the C-terminal hydrophobic domain.

In principle, the cNOS peptide can bind to the protein in two orientations. Using a specific nitroxide spin-labeled cNOS peptide derivative, we have shown that the C-terminal part of the cNOS peptide binds to the N-terminal hydrophobic surface of CaM; consequently, the N-terminal portion of the peptide binds to the C-terminal domain of CaM. This orientation resembles the complexes of CaM with the CaM-binding domains of MLCK (Ikura *et al.*, 1992; Meador *et al.*, 1992), CaM-kinase II (Meador *et al.*, 1993), and adenylate cyclase (Craescu *et al.*, 1995). From a sequence alignment of the cNOS peptide with the CaM-binding domains of MLCK, we find that two anchoring hydrophobic residues are found with a spacing of 14 residues, analogous to MLCK, but distinct from CaM kinase II. Moreover, isotope-edited difference FTIR studies have shown that the two domains of CaM retain their secondary structure upon binding the cNOS peptide, as well as the MLCK peptide (Zhang *et al.*, 1994b). Therefore, our data establish that the cNOS peptide binds to CaM in an analogous fashion as the MLCK peptide.

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