Circular Dichroism and $^1$H NMR Studies on the Structures of Peptides Derived from the Calmodulin-binding Domains of Inducible and Endothelial Nitric-oxide Synthase in Solution and in Complex with Calmodulin

NASCENT $\alpha$-HELICAL STRUCTURES ARE STABILIZED BY CALMODULIN BOTH IN THE PRESENCE AND ABSENCE OF Ca$^{2+}$

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There exist two types of nitric-oxide synthase (NOS); constitutive isoforms that are activated by binding calmodulin in response to elevated Ca$^{2+}$ and an inducible isozyme that binds calmodulin regardless of Ca$^{2+}$. To study the structural basis of the difference in Ca$^{2+}$ sensitivity, we have designed synthetic peptides of minimal lengths derived from the calmodulin-binding domain of endothelial NOS (eNOS) and that of macrophage NOS (iNOS). A peptide, RKEIPRLKVLKAVLFAALMRK, derived from human iNOS sequence, retained the ability to bind to calmodulin both in the presence and absence of Ca$^{2+}$, while a peptide derived from human eNOS sequence, RKKTFFKEVANAVKISASLMG, bound to calmodulin only in the presence of Ca$^{2+}$. Circular dichroism and two-dimensional $^1$H nuclear magnetic resonance studies suggested that both peptides assume nascent $\alpha$-helical structures in aqueous solution. When mixed with calmodulin, both peptides showed circular dichroism spectra characteristic for $\alpha$-helix. In contrast to other target proteins, the addition of iNOS peptide to calmodulin did not affect the Ca$^{2+}$ binding of calmodulin appreciably. The peptide derived from the calmodulin-binding domain of iNOS, therefore, binds in $\alpha$-helical structures both to Ca$^{2+}$-calmodulin and apo-calmodulin, which is unique among various target proteins of calmodulin.

NO$^1$ is a major messenger molecule playing key roles in

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The abbreviations used are: NO, nitric oxide; NOS, nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; CD, circular dichroism; TFE, trifluoroethanol; COSY, two-dimensional correlated spectroscopy; DQF-COSY, double quantum filtered COSY; $^{5,5'}$-Br$_2$BAPTA, $^{5,5'}$-dibromo-1,2-bis(2-amino-phenoxo)ethane-$N,N'$-$N''$-$N'''$-tetraacetic acid; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; MES, 4-morpholineethanesulfonic acid.

many physiological processes (1–3). NO production is catalyzed by at least three major isoforms of the NO synthase (NOS): type I (neuronal, nNOS) (4, 5), type II (inducible, iNOS) (6–8), and type III (endothelial, eNOS) (9). Although they share a common fundamental structure consisting of three well conserved domains, a cytochrome P-450-like heme protein domain, a cytochrome P-450 reductase-like flavoprotein domain, and a putative calmodulin-binding domain which connects the other two domains (5, 10, 11), their activities are regulated differently. Two isoforms, neuronal NOS and eNOS, are constitutively expressed and their activities are regulated by binding calmodulin in a response to elevated Ca$^{2+}$. In contrast, the third isozyme, iNOS, is rapidly induced in macrophages and in vascular smooth muscle cells after stimulation with bacterial lipopolysaccharides or cytokines, and remains active regardless of Ca$^{2+}$ (12). It has been demonstrated that calmodulin binds tightly to iNOS even in the absence of Ca$^{2+}$ (12).

A large number of the binding domains of calmodulin-bind- ing proteins have been shown to have a so-called basic amphiphilic $\alpha$-helix as a common motif (13, 14). There are no clear cut conserved amino acids among them, except that basic hydrophilic and hydrophobic amino acids appear alternately at certain intervals. When they assume $\alpha$-helical structures, the two groups of amino acids segregate on opposite sides of the helices. Formation of such helical structures, in fact, has been demonstrated in the three-dimensional structures of target peptide and Ca$^{2+}$-calmodulin complexes (15, 16). It is suggested that hydrophobic interactions of the two lobes of Ca$^{2+}$-calmodulin with the $\alpha$-helical rod of the peptides are important (15).

While Ca$^{2+}$ is usually necessary for the calmodulin-substrate protein interaction, some proteins such as GAP-43 (17), phosphorylase kinase (18), and Bordetella pertussis adenylyl cyclase (19), can interact with calmodulin even in the absence of Ca$^{2+}$. Although the presence of a so-called IQ motif among the target proteins that show Ca$^{2+}$-independent calmodulin bind- ing has been pointed out (20, 21), iNOS lacks such a motif. Using deletion-mutant and chimera proteins, the Ca$^{2+}$ insensitivity of iNOS has been clearly demonstrated to reside in the calmodulin-binding domain, although additional domains may play supportive roles (22–24). Therefore, a detailed study on the structural characteristics of the calmodulin-binding do- mains of various NOS isoforms is important to understand the structural basis underlying in the differential target recogni- tion of NOS isoforms by calmodulin. Although a number of
papers have recently appeared demonstrating the interaction of peptides derived from the putative iNOS calmodulin-binding domain with calmodulin (22, 25–27), all of the synthetic peptides suffer from the formation of large aggregates of calmodulin and peptides, which makes them unsuitable for detailed structural studies.

In the present study, we have designed synthetic peptides of minimal length derived from the calmodulin-binding domain of human iNOS and eNOS, which still retained the characteristics of the intact proteins in calmodulin binding but would not cause aggregation of calmodulin-peptide complexes. This enabled us to study their structures in solution and in calmodulin complex in detail. The results obtained showed that both peptides assume α-helical structures in aqueous solution and that the addition of calmodulin stabilizes these structures. The peptide derived from the iNOS sequence formed calmodulin complexes in α-helical conformation both with apo- and Ca$^{2+}$-calmodulin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Calmodulin purified from bovine brain was obtained from Wako Pure Chemical Industries, Ltd., Japan. Dansyl calmodulin was purchased from Sigma. Other chemicals used were of the highest grade commercially available. A 20-residue peptide corresponding to the calmodulin-binding domain of human eNOS, RKKTFKKEVANVKISA$^1$LMG (corresponding to residues 492–511), and a 23-residue peptide corresponding to the putative calmodulin-binding domain of human iNOS, KRRREIPKVLKVA$^1$FACLM$^1$R (residues 509–531), were synthesized with a standard t-Boc chemistry using an Applied Biosystems peptide synthesizer 430A and purified by reversed-phase high-performance liquid chromatography and electrospray mass spectrometry (28, 29). Peptide concentrations were determined by quantitative amino acid analysis.

**Circular Dichroism Spectroscopy**—Circular dichroism (CD) spectra were measured in a Jasco J-720 CD spectropolarimeter at 500 MHz. Chemical shifts were measured relative to the methyl resonance of 4,4-dimethyl-2-silapentane-1-sulfonate, used as an internal reference. Synthetic peptide (5 mM) was dissolved in 10% D$_2$O in H$_2$O, 99.98% D$_2$O, H$_2$O/D$_2$O/trifluoroethanol (TFE)-d$_3$ (5/1/4, v/v), or D$_2$O/TFE-d$_3$ (6/4). The pH of the samples was 4.0 (direct meter reading). The sequence-specific assignment of resonances was obtained from two-dimensional TOCSY, NOESY, DQF-COSY, and TQF-COSY spectra, as has been described previously (35). All spectra were acquired at 25 °C in the phase-sensitive mode using the time proportional phase increment technique, and were processed using Bruker XWIN-NMR or MSI Felix 95.0 software packages.

**RESULTS**

**Design of Peptides Derived from eNOS and iNOS Sequences Suitable for Structural Studies**—The amino acid sequences of the peptides synthesized in the present study are shown together with those reported in the previous studies (Table I). They correspond to the putative calmodulin-binding domains located in the middle of each NOS isozyme. While the peptide derived from eNOS sequence used in the present study was much shorter than the peptides used in the previous studies (22), the peptide corresponding to the iNOS sequence used in the present study was much shorter than the peptides used in the previous studies (22, 25–27). Long peptides derived from iNOS calmodulin-binding domain all bind to calmodulin with high affinities both in the presence and absence of Ca$^{2+}$ (25), a shorter peptide lacking 9 amino acids in the C terminus binds to calmodulin only very weakly even in the presence of Ca$^{2+}$ (22). However, the addition of the long peptides to calmodulin was analyzed with a JASCO FP-777 spectrophotometer in a 1 × 1-cm quartz cuvette. With the excitation wavelength set at 340 nm, emission spectra of dansyl calmodulin in the presence or absence of peptides were recorded in 150 mM Tris-HCl buffer (pH 7.5) containing 1 mM CaCl$_2$ or the indicated concentrations of EGTA. Binding of the peptides to calmodulin was monitored by recording the fluorescence emission at 490 nm. Dissociation constants of calmodulin-peptide complexes were determined by a direct fit of the data to the mass equation using a non-linear least square method (32).

**Determination of Ca$^{2+}$-Binding Constants**—Microscopic Ca$^{2+}$-binding constants of calmodulin were determined using 5,5′-Br$_2$BAPTA, as described (33). Buffers were treated with Chelex 100 resin (Bio-Rad) to remove Ca$^{2+}$. Calmodulin was decalcified by passing over a Sephadex PD-10 column (Pharmacia) and a Chelex 100 column (34). The residual Ca$^{2+}$ content was determined by atomic absorption spectroscopy (Hitachi Z-8100). Solutions containing calmodulin alone (10 μM), calmodulin (10 μM), and eNOS peptide (20 μM), or calmodulin (10 μM) and iNOS peptide (15 μM) were titrated with Ca$^{2+}$ in 10 mM MES-NaOH buffer (pH 6.8) containing 75 mM NaCl and 25 μM 5,5′-Br$_2$BAPTA at 25 °C. The individual microscopic binding constants (K$_{1a}$, K$_{1b}$) were obtained from least-squares fits directly to the titration data (33).

**Two-dimensional 1H NMR Analysis**—1H NMR spectra were recorded on a Bruker DMX-500 spectrometer operating at 500 MHz. Chemical shifts were measured relative to the methyl resonance of 4,4-dimethyl-2-silapentane-1-sulfonate, used as an internal reference. Synthetic peptide (5 mM) was dissolved in 10% D$_2$O in H$_2$O, 99.98% D$_2$O, H$_2$O/D$_2$O/trifluoroethanol (TFE)-d$_3$ (5/1/4, v/v), or D$_2$O/TFE-d$_3$ (6/4). The pH of the samples was 4.0 (direct meter reading). The sequence-specific assignment of resonances was obtained from two-dimensional TOCSY, NOESY, DQF-COSY, and TQF-COSY spectra, as has been described previously (35). All spectra were acquired at 25 °C in the phase-sensitive mode using the time proportional phase increment technique, and were processed using Bruker XWIN-NMR or MSI Felix 95.0 software packages.

**TABLE I**

Comparison of amino acid sequences of the iNOS- and eNOS-derived peptides

| Amino acid sequences | Species | +Ca$^{2+}$ | −Ca$^{2+}$ | Ref. |
|----------------------|---------|------------|------------|-----|
| a. iNOS peptides    |         |            |            |     |
| KRRREIPKVLKVAFFACLMK | Human   | 3.3        | 40–100     | This study |
| KLRPRREIRERLVKVFASLMKVRM | Murine | <1.0       | +a         | 25   |
| RREIRERLVKVFASLMKVRM | Murine  | <1.0       | +          | 25   |
| RREIRERLVKVFASLMKVRM | Murine  | +          | b          | 26   |
| RRREIPRREIRERLVKVFASLMKVRM | Murine | 1.2        |            | 22   |
| RREIRERLVKVFASLMKVRM | Murine  |            | −          | 22   |
| RREIRERLVKVFASLMKVRM | Murine  | <0.1       | +          | 27   |
| b. eNOS peptides    |         |            |            |     |
| RKKTFKKEVANVKISA$^1$LMG | Human | 2.9        |            | −    |
| TRKKTFKKEVANVKISA$^1$LMG | Bovine | 4.0        |            | 22   |

a. Binding was observed, but no quantitative data were presented. b. No binding was observed.
modulin has been shown to cause formation of large aggregates leading to precipitation of the peptide-calmodulin complex (25, 26), which would clearly hamper detailed structural studies aimed in the present study. We have, therefore, attempted to design a peptide which could mimic the calmodulin-binding characteristics of the intact protein but would not cause aggregation. The rationale for the present design is that they should retain the two large hydrophobic amino acids near both ends that contribute mostly in the interaction of several target peptides with Ca²⁺-calmodulin (underlined in Table I). Together with the 12 amino acids in between, they should form the core, but only a few amino acids outside the core contribute to the binding (15, 16). Addition of a longer sequence which itself has the characteristic basic amphiphilic nature of calmodulin-binding domain may create a second calmodulin-binding site in a single peptide, which could lead to the formation of large aggregates since calmodulin itself is bifunctional with its two independent lobes (26). Therefore, we added three residues C-terminal to Leu²⁰ and six amino acids N-terminal to Leu¹, most of them being hydrophilic amino acids. The N-terminal side was made slightly longer to increase the overall solubility of the peptide.

Stoichiometric binding of these peptides to calmodulin in the presence of Ca²⁺ was demonstrated by gel mobility shift assay (Fig. 1). Clear shifts in the mobility of calmodulin due to complex formation were observed with increasing amounts of the peptides, and almost complete shifts were observed at equimolar ratio with both peptides. The short iNOS peptide designed in the present study, therefore, retained the high affinity to calmodulin in the presence of Ca²⁺. When compared with the previous results (22), the addition of the two basic residues C-terminal to Met²¹ in the present peptide seems to increase significantly the affinity of the peptide to calmodulin. Interestingly, the directions of the mobility shifts were reversed with the two peptides under our gel conditions. Since the mobility shifts observed reflect a drastic conformational change of calmodulin upon binding of the target peptides (15, 16) in addition to an increase in apparent molecular weight, this suggests that the conformational changes of calmodulin upon binding of these two peptides are quite different. It should be noted that the loss of calmodulin due to formation of large aggregate reported with longer peptides (25, 26) was not observed, suggesting that the present short peptide did not cause aggregation under the conditions employed. On the contrary, such mobility shifts of calmodulin were not observed with both peptides in the presence of a Ca²⁺ chelator, EGTA (data not shown). However, the binding of the iNOS peptide to calmodulin in the absence of Ca²⁺ could be demonstrated by several techniques as will be described below.

Interaction of NOS Peptides with Calmodulin Studied by Fluorescence Spectroscopy—To demonstrate the complex formation, fluorescence change of dansyl calmodulin upon addition of the peptides was studied. Upon mixing with eNOS peptide in the presence of Ca²⁺, the fluorescence of dansyl calmodulin increased only slightly, but a shift of the maximum wavelength from 511 to 500 nm was clearly noted (Fig. 2a). The addition of iNOS peptide produced a more pronounced increase in the fluorescence intensity and a similar shift of the peak maximum (Fig. 2a). Interestingly, the fluorescence increase and shift of the maximum wavelengths observed with the two peptides were smaller than those observed with a calmodulin-binding domain peptide derived from MARCKS, a major in vivo substrate of protein kinase C. Under the same experimental conditions, the addition of the MARCKS-derived peptide to Ca²⁺-calmodulin increased the fluorescence intensity by more than 2-fold, and produced a greater shift of the maximum wavelength to 480 nm (data not shown). These observations suggest that the conformation of calmodulin in the complex with various target peptides differ significantly and affect the environment around the dansyl groups bound to the N terminus of calmodulin. It should be noted that the apparent “quenching” of fluorescence due to precipitation reported previously (25) was not observed, which enabled us to determine the dissociation constants directly.

When eNOS peptide was added to the dansyl calmodulin in the presence of EGTA, the fluorescence emission spectrum did not change significantly, suggesting that the peptide did not bind to calmodulin in the absence of Ca²⁺ (Fig. 2b). The interaction of the calmodulin-binding peptide of eNOS with calmodulin is clearly Ca²⁺ dependent as expected from the Ca²⁺ dependence of the isozyme (36, 37). In contrast, a drastic change in the fluorescence spectrum was observed when iNOS peptide was added to Ca²⁺-free dansyl calmodulin (Fig. 2b). The intensity increased by more than 2-fold, with a concomitant large shift in the maximum wavelength to 495 nm. These results not only demonstrate that the peptide of minimal length designed in the present study retain the ability to bind to calmodulin both in the presence and absence of Ca²⁺, but also suggest that the conformational change of calmodulin induced by the complex formation is more prominent in the absence of Ca²⁺ than in the presence of Ca²⁺.

Determination of Dissociation Constants by Fluorescence Spectroscopy—Since we could succeed in designing synthetic peptides suitable for detailed biophysical studies, fluorescence changes of dansyl calmodulin were used to determine the dissociation constants of the synthetic peptides and calmodulin. Fig. 3 shows the titration curves obtained when a fixed concentration of dansyl calmodulin (200 nM) was titrated with either eNOS or iNOS peptide in the presence or absence of Ca²⁺. In the presence of Ca²⁺, dissociation constants determined by the direct fit of the data to mass equation were 2.9 ± 1.4 nM (n = 6) and 3.3 ± 1.2 nM (n = 5) for eNOS peptide and iNOS peptide,
Effects of NOS Peptides on Ca$^{2+}$ -Binding of Calmodulin—Since the binding of the iNOS peptide to calmodulin observed in the absence of Ca$^{2+}$ could arise from the residual Ca$^{2+}$ due to the increase in the affinity of calmodulin to Ca$^{2+}$ in the presence of target peptides (38), we have determined the Ca$^{2+}$-binding constants in the presence of the NOS peptides under carefully controlled conditions. Solutions containing calmodulin alone, calmodulin and eNOS peptide, or calmodulin and iNOS peptide were titrated with Ca$^{2+}$ in the presence of chromophoric chelator, 5,5'-Br$_2$BAPTA (Fig. 4). From the titration curves, the macroscopic binding constants were calculated (Table II). As is evident in the curves and the calculated binding constants, the addition of eNOS peptide increased drastically the affinity of Ca$^{2+}$ to calmodulin. Similar increases in the binding constants have been reported with other target proteins (38, 39). In contrast, the addition of iNOS peptide did not affect the titration curve significantly, which was reflected in the small change in the products of two individual binding constants ($K_d$ and $K_C$). Interestingly, the strong cooperativity observed between the two Ca$^{2+}$-binding sites, which is reflected in the difference between $K_d$ and $K_C$ ($K_d > K_C$), disappeared, and the binding became rather sequential (33). These data indicate that the binding of the iNOS peptide to calmodulin does not cause an increase in the Ca$^{2+}$ affinity of calmodulin, which would produce an apparent Ca$^{2+}$-insensitivity in the calmodulin-iNOS peptide interaction. The iNOS-derived peptide, therefore, is unique among the calmodulin target peptides both in the high affinity to apo-calmodulin and in the lack of the modulation of Ca$^{2+}$-binding property of calmodulin.

Effects of TFE on Peptide Conformation—We have recently shown that eNOS peptide forms $\alpha$-helix in high concentrations of trifluoroethanol (TFE) (30). The reagent mimics hydrophobic environments and induces an $\alpha$-helical conformation, if peptides have a tendency to form the structure. To study the structural characteristics of the iNOS peptide, CD spectra of the peptide were measured under various conditions. As shown in Fig. 5, the CD spectrum of the iNOS peptide taken in aqueous buffer showed a single negative peak at around 202 nm, suggesting that the peptide does not have a distinct structure as described above. However, when compared with the CD spectrum of eNOS peptide, a shift of the peak maximum to longer wavelength and the appearance of a second broad peak at around 215-230 nm are notable. This indicated the presence of some $\alpha$-helical and/or $\beta$-sheet elements in the iNOS peptide structure. In 40% TFE solution, two negative peaks at 208 and 222 nm with a large positive peak at shorter wavelengths were observed, suggesting that the peptide took an $\alpha$-helical conformation. With increasing concentrations of TFE, the $\alpha$-helix content increased and reached a plateau at TFE concentrations above 30% (Fig. 5, inset). The concentration dependence of TFE-dependent spectral change of both peptides were very similar, and the final contents of $\alpha$-helix reached around 60% in both cases. Therefore, the calmodulin-binding domain of iNOS peptide could form an $\alpha$- helix like the corresponding domain of eNOS, when it is put under hydrophobic environments, such as in calmodulin complex or in phospholipid membranes (30). These features are common among various calmodulin-binding peptides and proteins (40, 41).

Structural Characteristics of iNOS and eNOS Peptides Studied by Two-dimensional $^1$H NMR—Structural characteristics of the peptides in aqueous solution were further analyzed by two-dimensional $^1$H NMR. For two-dimensional NMR data analysis, the standard method proposed by Wüthrich (42) was used to carry out the sequential assignment of all the proton resonances. TOCSY spectra were used to identify spin systems, and NOESY spectra were used to obtain inter-residual connectivities and distinguish equivalent spin systems. Data obtained with DQF-COSY spectra were used to confirm the assignments. DQF-COSY spectra of the NH-$\delta$H region for both peptides are shown in Fig. 6. We could assign most protons of both peptides even in aqueous solution. From these assignments, we calculated the difference between the NH proton chemical shifts in iNOS or eNOS peptide and those found in random coil as shown in Fig. 7. Since an $\alpha$-helical structure is indicated by upfield shifts of more than 0.1 ppm from typical chemical shift observed with random coil (42), these data suggest that both
peptides assume nascent \( \alpha \)-helical structures in aqueous solution.

Conformation of Peptides in Calmodulin Complex—To elucidate the underlying molecular mechanisms in the calmodulin-peptide interaction, we studied conformations of the peptides bound to calmodulin by CD spectroscopy. When eNOS peptide was added to calmodulin at a 1:1 ratio in the presence of Ca\(^{2+}\), the induction of a significant amount of \( \alpha \)-helical structure in the complex was noted (Fig. 8a). A similar change in CD spectrum was also observed with the addition of iNOS peptide to Ca\(^{2+}\)-calmodulin (Fig. 8a). It is generally assumed that Ca\(^{2+}\)-calmodulin itself does not gain secondary structure when bound to a target peptide, since only the central helix of calmodulin tends to accommodate the peptide bond (15, 16, 43). Therefore, the increased \( \alpha \)-helical contents can be attributed mainly to those of the target peptides. Structural characteristics of the peptides in calmodulin complex, therefore, are manifested in the difference spectra where the CD spectra of calmodulin alone are subtracted from those of the peptide-calmodulin complexes (Fig. 8b). They clearly show that the both peptides assume mainly \( \alpha \)-helical structures. Note that the negative peaks at 222 and 208 nm observed with eNOS peptide were deeper than those obtained with iNOS peptide, although the latter showed higher \( \alpha \)-helical content in aqueous solution.

When eNOS peptide was added to apo-calmodulin in the presence of EGTA, the CD spectrum of the mixture was different from that of the same mixture obtained in the presence of Ca\(^{2+}\) (Fig. 8c). The increase in the depth of the negative peak at around 222 nm was more significant, compared with that of the broad peak at shorter wavelengths. These characteristics are clearly seen in the difference spectrum, which indicates that the peptide is predominantly in a random coil structure similar to that of the peptide in aqueous buffer (Fig. 8d). This confirms again that the eNOS peptide does not form a complex with calmodulin in the absence of Ca\(^{2+}\). On the contrary, a marked change in CD spectrum was observed upon addition of iNOS peptide to apo-calmodulin in the presence of EGTA (Fig. 8c). The difference spectrum clearly shows the induction of an \( \alpha \)-helical structure in Cd-calmodulin complex (Fig. 8d). It should be noted that the depth of the negative peaks due to \( \alpha \)-helix observed with iNOS peptide in the absence of Ca\(^{2+}\) was almost twice compared with those observed in the presence of Ca\(^{2+}\), suggesting that the peptide assumes different conformations in the Ca\(^{2+}\)-calmodulin and apo-calmodulin complexes. These results suggest that the induction or stabilization of the \( \alpha \)-helical structure of the target protein by calmodulin is important for the interaction both in the presence and absence of Ca\(^{2+}\).

**DISCUSSION**

Since three NOS isoforms show drastic differences in their Ca\(^{2+}\)-sensitivity, the difference in molecular recognition mechanism among the isozymes is not only interesting from the structural point of view, but also vital for the understanding of the regulatory mechanisms of this important enzyme. Contrary to the neuronal NOS and eNOS isozymes that show a “normal” Ca\(^{2+}\)-dependent calmodulin binding, iNOS shows a unique Ca\(^{2+}\)-independent binding to calmodulin. A number of recent papers demonstrated that the putative calmodulin-binding domain of iNOS has the intrinsic ability to bind to calmodulin regardless of Ca\(^{2+}\) (22, 24–27). Except for a short 23-amino acid peptide (residues 501–523), various synthetic peptides derived from the iNOS sequence bind to both Ca\(^{2+}\)- and apo-calmodulin with high affinities (Table I). Contrary to the longer peptide used in the previous studies, our present peptide did not cause aggregation of calmodulin-peptide complex under conditions employed, but retained the ability to bind to calmodulin both in the presence and absence of Ca\(^{2+}\). This enabled us not only to study the structures of the peptides but also to determine directly the dissociation constants of the peptide to calmodulin both in the presence and absence of Ca\(^{2+}\). The obtained values are in the range of \( 10^{-9} \) M (in the presence of Ca\(^{2+}\)) to \( 10^{-7} \) M (in the absence of Ca\(^{2+}\)), which are high enough to bind to calmodulin tightly regardless of Ca\(^{2+}\). Therefore, the canonical calmodulin-binding domain of iNOS has the intrinsic ability to bind to calmodulin both in the presence and absence of Ca\(^{2+}\). The decrease in the affinity in the absence of Ca\(^{2+}\) by more than 1 order of magnitude can account for the partial dissociation of calmodulin from iNOS reported previously (26).

Recent results using the chimeric NOS and truncated mutants suggest that an additional domain within residues 484–726 is necessary for the binding of calmodulin in the absence of Ca\(^{2+}\) (24). The presence of two binding sites, therefore, may be responsible for the tight binding of iNOS protein to apo-calmodulin. In this context, it is of interest to note that the domain C-terminal to the canonical calmodulin-binding domain of iNOS has itself a basic amphiphilic character, and that all of the iNOS peptides longer than the present one suffered from the aggregation (25, 26). The synthetic peptide used in the present study which lacks most of the C-terminal domain did not show such tendency. The formation of large aggregates requires the presence of two binding sites in both reactants, i.e., calmodulin and the target-peptide. Since calmodulin has two

**TABLE II**

Macroscopic Ca\(^{2+}\) binding constants for the calmodulin in the absence and presence of the NOS peptides

| Sample          | \( \log(K_i) \) | \( \log(K_J) \) | \( \log(K_{iJ}) \) | \( \log(K_{iJ}) \) |
|-----------------|----------------|----------------|----------------|----------------|
| Calmodulin      | 4.8            | 7.1            | 5.0            | 5.5            |
| Calmodulin + eNOS peptide | 7.6            | 6.8            | 7.5            | 6.6            |
| Calmodulin + iNOS peptide | 6.4            | 5.9            | 5.0            | 5.4            |
independent lobes that can bind the target protein independently, this suggests that the synthetic peptides containing both the canonical calmodulin-binding domain and the domain C-terminal to that should be divalent and each site can bind to each lobe of calmodulin independently (26). Whether such binding modes exist in the interaction of intact iNOS with calmodulin is, of course, still to be elucidated.

Although CD spectra indicated only random structures, two-dimensional $^1$H NMR studies suggested that the both peptides derived from the canonical calmodulin-binding domains of eNOS and iNOS assumed nascent $\alpha$-helical structures in aqueous solution. As reported for the calmodulin-binding peptides of GAP-43 (35) and neuronal NOS (37), such a nascent $\alpha$-helical structure is important to adopt a regular $\alpha$-helical conformation. CD and NMR spectra of the peptides obtained in TFE solution indicate that such $\alpha$-helical structures are further stabilized under hydrophobic environments. We have previously shown that the calmodulin-binding domains of the three NOS isozymes act as membrane-binding domains, and that the binding of phospholipids induces $\alpha$-helical conformations of these domains (30). When bound to Ca$^{2+}$-calmodulin, both peptides derived from iNOS and eNOS calmodulin-binding domains assume $\alpha$-helical structures, as is the case for most of the calmodulin-binding domains of the target proteins (15). In the absence of Ca$^{2+}$, however, only the iNOS peptide showed a spectral change characteristic for an $\alpha$-helical conformation. Therefore, the calmodulin-binding domain of iNOS has the ability to bind in an $\alpha$-helical structure both to Ca$^{2+}$-calmodulin and apo-calmodulin, which is unique among various calmodulin-binding proteins.

GAP-43 and other proteins containing so-called IQ motif (IQXXRGXXXR) have been shown to bind calmodulin regardless of Ca$^{2+}$ (17, 20, 21). Recent studies have demonstrated that the overall structure of apo-calmodulin does not change upon interaction with GAP-43 peptide and that the exposed acidic residues rather than hydrophobic residues of apo-calmodulin are involved in the interaction with the peptide (44, 45). In the case of the iNOS peptide, such IQ motif is not present, and the affinity to calmodulin is higher by 2 or 3 orders of magnitude than that of GAP-43 both in the presence and absence of Ca$^{2+}$ (17). As will be published elsewhere, a calorimetric analysis on the binding reaction of iNOS peptide to calmodulin showed that the binding reaction is endothermic.

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**FIG. 6.** $^1$H (F1)/NH (F2) region of 500 MHz DQF-COSY spectra of NOS peptides in aqueous solution. DQF-COSY spectra of iNOS peptide (a) and eNOS peptide (b) were obtained in 90% H$_2$O-10% D$_2$O solution as described under “Experimental Procedures.” Concentrations of the peptides were 5 mM. Sequence specific assignments of the $^1$H/NH cross-peaks are indicated. Several residues whose signals were not individually assigned due to overlaps are circled.

**FIG. 7.** Deviation in chemical shifts of $^1$H protons from random coil. Deviations of chemical shifts of $^1$H protons observed with the NOS peptides from those obtained for random coil (42) are shown.

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$^2$ M. Matsubara and H. Taniguchi, manuscript in preparation.
with calmodulin considerably different from the previously known domains. The availability of peptides, which are suitable for biophysical analyses, should now facilitate the elucidation of the underlying mechanisms at molecular level.

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FIG. 8. CD spectra of calmodulin-peptide complex in the presence or absence of Ca2+. Concentrations of calmodulin and peptides were 10 μM in 10 mM MES buffer (pH 6.8) containing 75 mM NaCl and 1 mM CaCl2 or 2 mM EGTA. a, in the presence of 1 mM CaCl2, calmodulin alone (●), calmodulin plus iNOS peptide (□), calmodulin and eNOS peptide (△), or of calmodulin and eNOS peptide (□), and b, difference spectra were calculated by subtracting the spectrum of calmodulin alone from that of calmodulin and iNOS peptide (□). c, or of calmodulin and eNOS peptide (□), and d, difference spectra calculated by subtracting the CD spectrum of calmodulin alone from that of calmodulin plus iNOS peptide (□), or that of calmodulin plus eNOS peptide (△).