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Chemical Shift perturbations induced by residue specific mutations of CaM interacting with NOS peptides

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Abstract

The regulation of Nitric oxide synthase (NOS) by calmodulin (CaM) plays a major role in a number of key physiological and pathological processes. A detailed molecular level picture of how this regulation is achieved is critical for drug development and for our understanding of protein regulation in general. CaM is a small acidic calcium binding protein and is required to fully activate NOS. The exact mechanism of how CaM activates NOS is not fully understood at this time. Studies have shown CaM to act like a switch that causes a conformational change in NOS to allow for the electron transfer between the reductase and oxygenase domains through a process that is thought to be highly dynamic. The interaction of CaM with NOS is modified by a number of post-translation modifications including phosphorylation. Here we present backbone and sidechain ^{1}H, ^{15}N NMR assignments of modified CaM interacting with NOS peptides which provides the basis for a detailed study of CaM-NOS interaction dynamics using ^{15}N relaxation methods.

Keywords

Calmodulin. Nitric Oxide Synthase. NMR spectroscopy. Resonance assignment.

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Biological context

Calmodulin (CaM) is a ubiquitous cytosolic Ca^{2+}-binding protein that binds and regulates hundreds of different proteins (Ikura and Ames 2006). CaM consists of two globular domains, each containing two EF hand pairs capable of binding to Ca^{2+}. These are joined by a flexible central linker region. The binding of Ca^{2+} causes a conformational change of CaM that exposes hydrophobic patches on each domain thereby allowing CaM to associate with its intracellular target proteins. CaM's central linker's flexibility allows it to adapt its conformation to optimally associate with its intracellular targets (Persechini and Kretsinger 1988). There is considerable interest in obtaining a better understanding of the structural basis for CaM's ability to bind and recognize its numerous target proteins.

Nitric oxide synthase (NOS) enzymes are one of the target proteins bound and regulated by CaM. These enzymes catalyze the production of nitric oxide (•NO) that acts as a secondary inter- and intracellular messenger involved in many physiological processes (Alderton et al. 2001). There are three NOS isozymes found in mammals: neuronal NOS (nNOS, NOS I), endothelial NOS (eNOS, NOS III), and inducible (iNOS, NOS II). The NOS enzymes are homodimeric with each monomer containing an *N*-terminal oxygenase domain and a *C*-terminal reductase domain, connected by a CaM binding domain. CaM binding is required for efficient electron transfer from the reductase to the oxygenase domain for •NO production (Alderton et al. 2001, Daff 2010). The large conformational change that CaM induces in the reductase domain of the NOS enzymes allows for the FMN domain to interact with both the FAD to accept electrons and pass the electrons on to the heme during catalysis (Welland and Daff 2010). Clearly, these conformational changes caused by CaM are important in stimulating efficient electron transfer within the NOS enzymes.

The interaction of CaM with its target proteins is modified by a number of post-translation modifications including phosphorylation (Jang et al. 2007). Tyrosine 99 of CaM is known to be phosphorylated by four tyrosine kinases. This phosphorylation has been reported to affect the CaM-dependent activity of a number of enzymes including the NOS enzymes (Corti et al. 1999, Mishra et al. 2009). Studies of central nervous tissue hypoxia in newborn piglets indicated that phosphorylation of

Y99 of CaM affect the activity of NOS in vivo (Mishra et al. 2009). The helix-2 – helix-6 region (latch domain) of CaM is also an important interaction site between CaM and NOS and plays a critical role in NOS activation (Meador et al. 1992, Zenghua et al. 1995). Amino acid mutations in this site have been shown to impair activation of the NOS enzymes also (Zenghua et al. 1995). To allow the structural studies to be performed, a phosphomimetic form of CaM, CaM Y99E, and CaM containing Y99E and a N111D latch domain mutations were used in the investigation.

Here we present the NMR resonance assignments of CaM with a phosphomimetic mutation at Y99 interacting with eNOS and CaM with a phosphomimetic mutation at Y99 and latch domain mutation at N111 interacting with iNOS. The NMR resonance assignments of wild type CaM bound to the iNOS and eNOS CaM binding domain peptides have previously been determined (BMRB 18027 and 18028). By comparing to the wild type complexes we clearly show that phosphorylation of CaM and mutation of a latch domain residue causes slight perturbations of resonance assignments for residues near the mutation sites and involved in the C-terminal Ca^{2+} binding sites, whereas the N-terminal and linker region residues appear unaffected. Furthermore, this method allows for quick structural characterization of other CaM or CaM mutants interacting with various NOS peptides and provides the basis for a detailed study of CaM-NOS interaction dynamics using ^{15}N relaxation methods.

Methods and experiments

Expression and purification of CaM Y99E and CaM Y99E N111D

The QuikChange site-directed mutagenesis procedure was used to produce vectors coding for CaM Y99E and CaM Y99E N111D in the kanamycin resistant pET9dCaM plasmid. The human iNOS (RREIPLKVLVKAVLFACMLMRK, 22 residues corresponding to residues 510-531 from the full length iNOS protein) and eNOS (TRKKTFKEVANAVKISASLMGT, 22 residues corresponding to residues 491-512 from the full length eNOS protein) peptides were synthesized and purchased from Sigma. CaM Y99E and CaM Y99e N111D were expressed in *E. coli* BL21DE3 competent cells and grown in M9 media (11.03 g/L $Na_2HPO_4 \cdot 7H_2O$, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 5 mg/mL Thiamine, 100 μg/mL kanamycin) containing 2 g/L glucose and 1 g/L $^{15}NH_4Cl$ at

37°C. Protein expression was induced at an OD_{600nm} of 0.6 with 500 μM isopropyl-*β*-D-thiogalactopyranoside (IPTG) and harvested after 4 h by centrifugation at 6000 x g at 4°C for 5 minutes. Cells were resuspended in 4 volumes of 50 mM MOPS, 100 mM KCl, 1 mM EDTA, 1 mM DTT, pH 7.5 and lysed by homogenization using an Avestin EmulsiFlex-C5 homogenizer (Ottawa, ON). The lysate was then clarified by centrifugation at 48,000 x g for 30 minutes at 4°C. To the clarified supernatant, $CaCl_2$ was added to a concentration of 5 mM in order to saturate CaM with Ca^{2+} and induce the exposure of hydrophobic patches in the N- and C-domains of CaM to allow CaM to interact with the resin. This Ca^{2+}-saturated supernatant was then loaded onto 20 mL of phenyl sepharose 6 fast flow highly-substituted resin (GE Healthcare Bio-Sciences, Baie d'Urfe, PQ) in a 1 cm x 30 cm Econo-column (Bio-Rad Laboratories, Mississauga, ON) equilibrated with 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.5 @ 4°C. After the Ca^{2+}-saturated solution was loaded; the resin was washed with 100 mL of the above. The resin was subsequently washed with 80 mL of 50 mM Tris-HCl, 500 mM NaCl, 1 mM $CaCl_2$, pH 7.5 @ 4°C to remove any non-specific proteins that were interacting with the resin. The resin was finally washed with 50 mL of 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.5 to remove NaCl from the resin. CaM was then eluted from the phenyl sepharose resin with approximately 30 mL of 10 mM Tris-HCl, 10 mM EDTA, pH 7.5 @ 4°C and 2 mL fractions were collected. Fractions were then scanned from 325 to 250 nm on a Varian Cary UV–visible Spectrophotometer (Varian, Mississauga, ON). Fractions displaying the characteristic absorbance peaks of CaM at 277 nm (for tyrosine residues) and 269, 265, 259, and 253 nm (for phenylalanine residues) were pooled and dialysed overnight against 1 L of 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.5 using 6-8000 MWCO dialysis tubing (VWR International, Mississauga, ON). Isolation and purity of the CaM proteins (148 residues) were confirmed by ESI-MS and SDS-PAGE.

NMR spectroscopy

The samples were prepared for NMR experiments via a buffer exchange into 100 mM KCl, 10 mM $CaCl_2$, 0.2 mM NaN_3, 90% H_2O/10% ^2H_2O at pH 6.0 using a YM10 centrifugal filter device (Millipore Corp., Billerica, USA). All NMR samples contained at least 1 mM CaM Y99E or CaM Y99E N111D in

a total volume of 500 μL. The samples were transferred into 5 mm NMR sample tubes and stored at 4^{o}C until required for NMR experiments. NMR experiments on the complexes were conducted on samples titrated with either iNOS or eNOS peptide to saturation in a 1:1 CaM:peptide ratio. Complex formation was monitored after each addition by acquisition of a ^{1}H-^{15}N heteronuclear single-quantum coherence (HSQC) spectrum.

NMR spectra were recorded at 298K on Bruker 600 and 700 MHz DRX spectrometers equipped with XYZ-gradients triple-resonance probes (Bruker, Billerica, MA, USA). Specific NMR resonance assignments were achieved using [^{1}H-^{15}N]-HSQC, HC(C)H-TOCSY, 15N-TOCSY-HSQC and ^{15}N-NOESY-HSQC experiments, while using the NMR resonance assignments of the wild type complexes as a starting point. Spectra were analyzed using the program CARA (Keller 2005).

Assignments and data deposition

Figure 1 shows the superposition of the [^{1}H,^{15}N]- HSQC spectra of the CaM Y99E bound to the eNOS CaM binding domain peptide versus wild type CaM bound to the eNOS CaM binding domain peptide. Chemical shift changes induced by the phosphomimetic mutant appear for the amides in the *C*-domain, specifically the amides of residues 96-101 and 130 - 138. Almost all of these residues participate in coordinating the Ca^{2+} ion in EF hands III and IV. The rest of the amide resonances overlay quite well with each other suggesting a similar structure of the N-domain for both complexes.

Almost complete amide resonance assignment for CaM Y99E bound to the eNOS CaM binding domain peptide was achieved. Overall 97.9% of all $^{1}H^{N}$, ^{15}N resonances were assigned with the exception of the first A1 residue, D129, E140 and the two Proline residues. The chemical shift assignment of CaM Y99E with eNOS was deposited in the BMRB database under accession number 25257.

Figure 2 shows the superposition of the [^{1}H,^{15}N]- HSQC spectra recorded at 298 K on the CaM Y99E N111D bound to the iNOS CaM binding domain peptide versus wild type CaM bound to the iNOS CaM binding domain peptide. Chemical shift changes induced by the phosphomimetic and latch domain mutant appear for the amides in the *C*-domain, specifically the amides of residues 96-103, 110 - 115 and

130 - 138. These residues participate in coordinating the Ca^{2+} ion in EF hands III and IV and are part of helix 6 of the latch domain. Surprisingly the rest of the amide resonances overlay quite well with each other suggesting a similar structure of the N-domain for both complexes, including the residues of helix 2 which are part of the helix 2-helix 6 latch domain.

Almost complete amide resonance assignment for CaM Y99E N111D bound to the iNOS CaM binding domain peptide was achieved. Overall 98.6% of all $^{1}H^{N}$, ^{15}N resonances were assigned with the exception of the first A1 residue, E120 and the two Proline residues. The chemical shift assignment of CaM Y99E N111D with iNOS was deposited in the BMRB database under accession number 25253.

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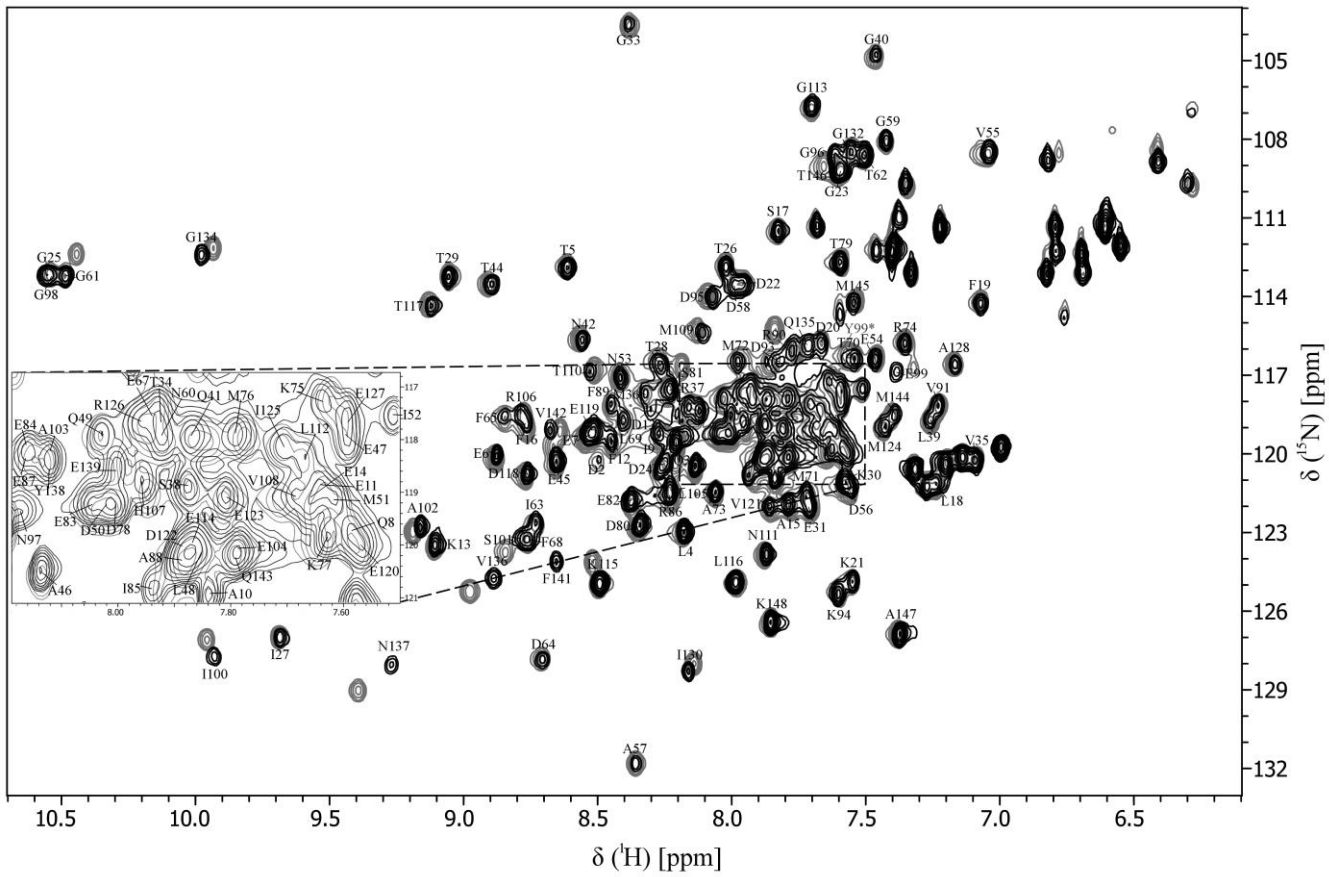

Fig. 1

Superposition of [^{1}H,^{15}N]-HSQC spectra of a CaM Y99E bound to the eNOS CaM binding domain peptide (black) and wild type CaM bound to the eNOS CaM binding domain peptide (grey). Each backbone amide resonance is labeled with the amino acid type and position in the sequence.

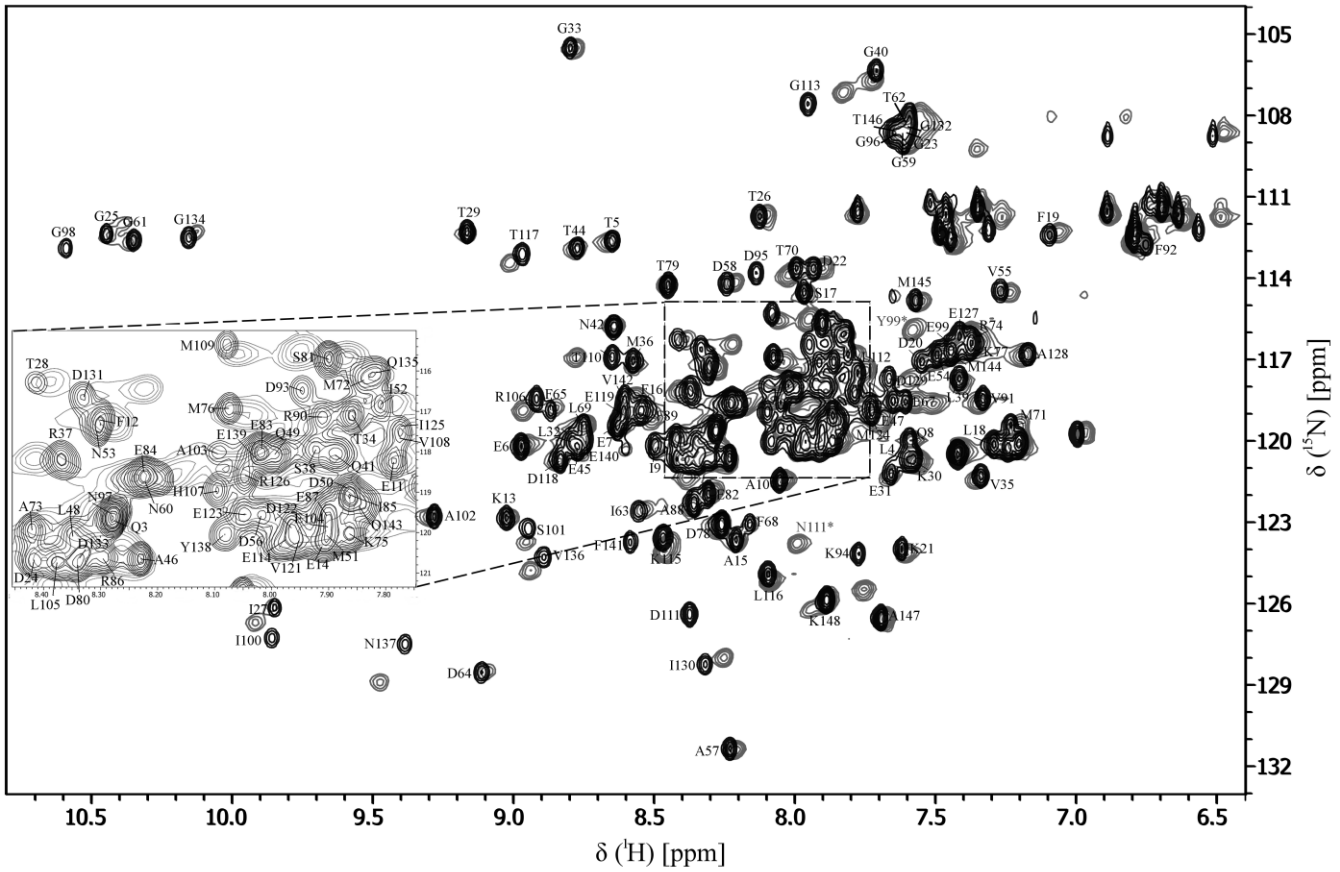

Fig. 2

Superposition of [^{1}H,^{15}N]-HSQC spectra of a CaM Y99E N111D bound to the iNOS CaM binding domain peptide (black) and wild type CaM bound to the iNOS CaM binding domain peptide (grey). Each backbone amide resonance is labeled with the amino acid type and position in the sequence.