

Abstract  
Ca$^{2+}$-Calmodulin binding to the variable N-terminal region of the diacylglycerol/phorbol ester-binding UNC13/Munc13 family of proteins modulates the short-term synaptic plasticity characteristics in neurons. Here, we report the sequential backbone and side chain resonance assignment of the Ca$^{2+}$-Calmodulin/Munc13-1458–492 peptide complex at pH 6.8 and 35°C (BMRB No. 15470).

Keywords  
Calcium · Calmodulin · Munc13 · Neurotransmitter release · Synaptic plasticity · Phorbol esters and vesicle priming

Biological context  
UNC13/Munc13 family of proteins are diacylglycerol/phorbol ester-binding proteins specifically localized to presynaptic active zones, where the synaptic vesicles dock and release their neurotransmitters to the intersynaptic cleft for the chemical signal transmission among neurons (Junge et al. 2004; Brose et al. 1995). In Caenorhabditis elegans, unc-13 is essential for coordinated movement (Brenner 1974). At a molecular level, UNC13/Munc13 proteins are essential in the maturation process of synaptic vesicles, remodeling the SNARE complex of proteins leading to fusion competent molecular states. Upon deletion of UNC13/Munc13 proteins neurotransmitter release is completely impaired (Richmond et al. 2001).

Ca$^{2+}$-Calmodulin (CaM) is a highly conserved ubiquitous eukaryotic signaling protein, regulating the activity of numerous proteins like protein kinases, phosphodiesterases, ion channels and pumps, and nitric-oxide synthases. Various studies have shown that CaM activates these proteins interacting with regulatory elements possessing an amphiphilic $\alpha$-helix character (Crivici and Ikura 1995).

It has been shown (Junge et al. 2004) that some of the Munc13 protein isoforms contain a highly conserved CaM binding motif in their variable N-terminal region. However, this binding motif does not belong to any of the classical CaM binding motifs previously described (Rhoads and Friedberg 1997). Synthetic peptides of the Munc13-1 and ubMunc13-2 isoforms belonging to this CaM binding domain form high-affinity complexes with CaM in vitro. To unravel the CaM binding mechanism to Munc13 proteins, we carried out NMR experiments on the CaM/Munc13-1458–492 peptide complex.

Methods and experiments  
NMR sample preparation  
The costs of $^{15}$N, $^{13}$C-uniformly labeled synthetic peptides are prohibitive. Therefore, we chose a recombinant approach...
to obtain the Munc13-1\textsuperscript{458–492} peptide and will be described elsewhere. Briefly, the CaM binding domain (458–492) coding sequence of \textit{munc13-1} was cloned into the pGEX-2T expression vector (GE Healthcare). The uniformly labeled (\textsuperscript{15}N, \textsuperscript{13}C) GST-Munc13-1\textsuperscript{458–492} fusion protein was overexpressed in M9 minimal medium containing \textsuperscript{15}NH\textsubscript{4}Cl and \textsuperscript{13}C-glucose as solely source of nitrogen and carbon, respectively. Due to solubility problems, the GST-Munc13-1\textsuperscript{458–492} fusion protein was co-expressed with CaM using resistance to two different antibiotics. The GST-Munc13-1\textsuperscript{458–492}/CaM complex was co-purified via affinity chromatography using GST-Sepharose (GE-Healthcare) according to the vendor instructions. The GST fusion was cleaved with thrombin and the Munc13-1 peptide purified by reverse-phase HPLC. The eluted peptide was lyophilized, reconstituted with 250 \mu l of the NMR-sample aqueous buffer (20 mM Bis–Tris, 150 mM KCl, 10 mM CaCl\textsubscript{2}, pH 6.8) and mixed with a 1.2 M excess of unlabeled CaM dissolved in (Millipore) water to give a total final volume of 2 ml. The CaM/Munc13-1\textsuperscript{1458–492} complex was concentrated by vacuum centrifugation in a Speedvac to a final volume of 250 \mu l giving a final concentration of approximately 0.5 mM. In order to get a lock signal in the NMR spectrometer, we added 10 \mu l of 99\% D\textsubscript{2}O.

The (U-\textsuperscript{15}N, \textsuperscript{13}C) labeled CaM was obtained as described (Guerini et al. 1984; Haberz et al. 2006). Briefly, CaM was over-expressed from a pET28a-CaM construct in \textit{E. coli} BL21(DE3) in labeled M9 minimal medium. The cells were lysed by ultrasound sonication and the cell debris removed by centrifugation at 15,000 g for 45 min. The supernatant was precipitated with 2.84\% trichloroacetic acid and subjected to hydrophobic interaction chromatography on a phenylsepharose column; CaM was eluted with an EGTA containing buffer. To prepare the complex sample, 8 mg of lyophilized (U-\textsuperscript{15}N, \textsuperscript{13}C) labeled CaM were weighted and dissolved in 2 ml of (Millipore) water. Around 2.4 mg of a synthetic natural abundance Munc13-1\textsuperscript{1459–492} peptide (1.2 equivalents) were weighted and dissolved in 250 \mu l of the NMR-sample aqueous buffer (20 mM Bis–Tris, 150 mM KCl, 10 mM CaCl\textsubscript{2}, pH 6.8) and added to the CaM solution; the mixture was concentrated by vacuum centrifugation in a Speedvac to give a final volume of 250 \mu l. 10 \mu l of 99\% D\textsubscript{2}O were added for the NMR-lock signal. An additional (U-\textsuperscript{15}N, \textsuperscript{13}C) labeled CaM/Munc13-1\textsuperscript{1459–492} complex sample dissolved in 99\% D\textsubscript{2}O was prepared in the same manner. The three samples were placed in 280 \mu l Shigemi microcells covered with their glass plunger and sealed with Parafilm.

NMR experiments

All NMR experiments were carried out at 35\degree C on the following \textit{Bruker} spectrometers: \textit{Avance} 600 MHz equipped with a \textit{z}-gradient HCN probe head; \textit{DRX} 600 MHz with a \textit{z}-gradient HCN probe head; \textit{Avance} 700 MHz with a triple-axis gradient HCN probe head; \textit{DMX} 800 MHz with a \textit{z}-gradient cryogenic HCN probe head; and \textit{Avance} 900 MHz with a \textit{z}-gradient cryogenic HCN probe head.

The experiments made use of pulse field gradient coherence selection. Quadrature detection in the indirect dimensions was achieved either with the States-TPPI or Echo/antiecho methods. The chemical shift referencing was achieved internally by the addition of 0.4 mM DSS. The acquisition parameters for the experiments are given in Table 1. The spectra were processed with XWIN-NMR, Felix (Accelerys, Inc.), NMR-PIPE (Delaglio et al. 1995) and analyzed with SPARKY (Goddard and Kneller 1999).

Assignments and data deposition

The sequential backbone and side chain resonance assignment is nearly complete and had been deposited in the Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu) under the accession number 15,470. The \textit{\textsuperscript{1}H-\textsuperscript{15}N-HSQC} spectrum of the (U-\textsuperscript{15}N, \textsuperscript{13}C) CaM/Munc13-1\textsuperscript{1459–492} complex sample is shown in Fig. 1. Its general appearance is consistent with that one of Ca\textsuperscript{2+}-CaM, however, many NH cross-peaks in the N-terminal domain (1–76) of CaM had large line-widths mainly in helices A and D. This gave rise to an assignment completeness of 95.7\% for the backbone resonances (CA, CB, C, N, H, HA and HB). In contrast, the assignment of the C-terminal domain (83–148) of the Ca\textsuperscript{2+}-CaM/peptide complex was nearly complete (98.8\% Backbone resonances). This suggested that the N-terminal domain of Ca\textsuperscript{2+}-CaM experiences conformational exchange, whereas the C-terminal domain consists of a single conformer under these conditions. In Fig. 2, the \textit{\textsuperscript{1}H-\textsuperscript{15}N-HSQC} spectrum of the (U-\textsuperscript{15}N, \textsuperscript{13}C) CaM/Munc13-1\textsuperscript{1458–492}/CaM complex is shown. An overlay of this spectrum with that one of a (U-\textsuperscript{15}N, \textsuperscript{13}C)-Munc13-1\textsuperscript{1458–492} peptide in 8 M urea solution (supplementary material) clearly revealed an increase in the amide resonance dispersion of the peptide, characteristic of secondary structure formation in this amphiphilic \textit{\alpha}-helix motif upon complex formation with Ca\textsuperscript{2+}-CaM.

The assignment of the labeled Munc13-1\textsuperscript{1458–492} peptide in complex with Ca\textsuperscript{2+}-CaM was completed to 99.2\% of the backbone resonances. The chemical shifts of the \textit{\alpha}-methyl protons in the methionines of Ca\textsuperscript{2+}-CaM in complex with the Munc13-1\textsuperscript{1458–492} peptide were compared with those of free Ca\textsuperscript{2+}-CaM. Most of the \textit{\alpha}-methyl protons of the eight methionines in Ca\textsuperscript{2+}-CaM were shifted upfield upon binding to the peptide. Significant changes (>0.15 ppm) were found for M51 (−0.3 ppm), M71 (−0.31 ppm), M109
Table 1  NMR data acquisition parameters

| Sample | Experiment | Spectral frequency MHz | Complex points |
|--------|------------|------------------------|----------------|
| (U-\(^{15}\)N, \(^{13}\)C) CaM/Munc13-1\(^{459-492}\) in 90% H\(_2\)O, 10% D\(_2\)O | \(^{1}\)H-\(^{15}\)N HSQC | 900 | 2,048 128 – |
|        | \(^{1}\)H-\(^{15}\)N NOESY-HSQC, \(t_{\text{mix}}\) 100 ms | 900 | 2,048 48 128 |
|        | HNCO       | 700                    | 2,048 46 60 |
|        | HN(CA)CO   | 600                    | 1,024 40 25 |
|        | HNCA/CB    | 700                    | 1,024 24 40 |
|        | CBCA(CO)NH | 600                    | 1,024 24 40 |
|        | H(CC)(CO)NH-TOCSY | 700 | 2,048 30 64 |
| (U-\(^{15}\)N, \(^{13}\)C) CaM/Munc13-1\(^{459-492}\) in 99% D\(_2\)O | \(^{1}\)H-\(^{13}\)C HSQC \(t_{\text{mix}}\) 120 ms | 700 | 2,048 48 58 |
|        | HCCH-TOCSY | 600                    | 2,048 64 128 |
|        | \(^{1}\)H-\(^{13}\)C HSQC | 700 | 2,048 128 – |
|        | \(^{1}\)H-\(^{13}\)C NOESY-HSQC (aromatic region) \(t_{\text{mix}}\) 130 ms | 700 | 2,048 48 40 |
|        | HBCB(Caro)HG | 600 | 2,048 46 – |
|        | HBCB(Caro)HE | 600 | 2,048 46 – |
| (U-\(^{15}\)N, \(^{13}\)C) Munc13-1\(^{458-492}\)/CaM in 90% H\(_2\)O, 10% D\(_2\)O | \(^{1}\)H-\(^{15}\)N HSQC | 600 | 1,024 128 – |
|        | \(^{1}\)H-\(^{15}\)N NOESY-HSQC and \(^{1}\)H-\(^{13}\)C NOESY-HSQC shared version, \(t_{\text{mix}}\) 120 ms | 600 | 2,048 180 114 |
|        | HNCO       | 900                    | 1,024 64 32 |
|        | HN(CA)CO   | 600                    | 1,024 46 32 |
|        | HNCA/CB    | 600                    | 1,024 50 100 |
|        | CBCA(CO)NH | 600                    | 1,024 29 60 |
|        | H(CC)(CO)NH-TOCSY | 600 | 1,024 26 50 |
|        | (H)CC(CO)NH-TOCSY | 600 | 1,024 30 52 |
|        | HCCH-TOCSY | 600                    | 2,048 32 128 |

Fig. 1 \(^{1}\)H-\(^{15}\)N-HSQC spectrum of the (U-\(^{15}\)N, \(^{13}\)C) CaM/Munc13-1\(^{459-492}\) complex measured at 35°C at 900 MHz. The highly overlapped central region is enlarged on the right side.
The effect on the chemical shift for the methionines is attributed to deshielding effects caused by the aromatic rings of the various aromatic amino acids present in the peptide complexed to Ca\(^{2+}\)-CaM.

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**Ethical standards** The experiments comply with the german current laws for ethical standards.

**Conflict of interests** The authors declare that they have no conflict of interest.

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**Fig. 2** \(^1\)H-\(^{15}\)N-HSQC spectrum of the (U-\(^{15}\)N, \(^{13}\)C) Munc13-1458–492/CaM complex recorded at 35°C at 600 MHz