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ARTICLE

^{1}H, ^{13}C and ^{15}N resonance assignments of the Calmodulin-Munc13-1 peptide complex

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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-$1^{458–492}$ 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 α-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-$1^{458–492}$ 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

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to obtain the Munc13-1$^{458–492}$ peptide and will be described elsewhere. Briefly, the CaM binding domain (458–492) coding sequence of *munc13-1* was cloned into the pGEX-2T expression vector (*GE Healthcare*). The uniformly labeled (^{15}N, ^{13}C) GST-Munc13-1$^{458–492}$ fusion protein was over-expressed in M9 minimal medium containing $^{15}NH_4Cl$ and ^{13}C-glucose as solely source of nitrogen and carbon, respectively. Due to solubility problems, the GST-Munc13-1$^{458–492}$ fusion protein was co-expressed with CaM using resistance to two different antibiotics. The GST-Munc13-1$^{458–492}$/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 µl of the NMR-sample aqueous buffer (20 mM Bis–Tris, 150 mM KCl, 10 mM $CaCl_2$, 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$^{459–492}$ complex was concentrated by vacuum centrifugation in a *Speedvac* to a final volume of 250 µl giving a final concentration of approximately 0.5 mM. In order to get a lock signal in the NMR spectrometer, we added 10 µl of 99% D_2O.

The (U-^{15}N, ^{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 *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-^{15}N, ^{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$^{459–492}$ peptide (1.2 equivalents) were weighted and dissolved in 250 µl of the NMR-sample aqueous buffer (20 mM Bis–Tris, 150 mM KCl, 10 mM $CaCl_2$, 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 µl. 10 µl of 99% D_2O were added for the NMR-lock signal. An additional (U-^{15}N, ^{13}C) labeled CaM/Munc13-1$^{459–492}$ complex sample dissolved in 99% D_2O was prepared in the same manner. The three samples were placed in 280 µl *Shigemi* microcells covered with their glass plunger and sealed with *Parafilm*.

NMR experiments

All NMR experiments were carried out at 35°C on the following *Bruker* spectrometers: *Avance* 600 MHz equipped with a *z*-gradient cryogenic HCN probe head; *DRX* 600 MHz with a *z*-gradient HCN probe head; *Avance* 700 MHz with a triple-axis gradient HCN probe head; *DMX* 800 MHz with a *z*-gradient cryogenic HCN probe head; and *Avance* 900 MHz with a *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 ^{1}H-^{15}N-HSQC spectrum of the (U-^{15}N, ^{13}C) CaM/Munc13-1$^{459–492}$ complex sample is shown in Fig. 1. Its general appearance is consistent with that one of Ca^{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^{2+}-CaM/peptide complex was nearly complete (98.8% Backbone resonances). This suggested that the N-terminal domain of Ca^{2+}-CaM experiences conformational exchange, whereas the C-terminal domain consists of a single conformer under these conditions. In Fig. 2, the ^{1}H-^{15}N-HSQC spectrum of the (U-^{15}N, ^{13}C) Munc13-1$^{458–492}$/CaM complex is shown. An overlay of this spectrum with that one of a (U-^{15}N, ^{13}C)-Munc13-1$^{458–492}$ 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 α-helix motif upon complex formation with Ca^{2+}-CaM.

The assignment of the labeled Munc13-1$^{458–492}$ peptide in complex with Ca^{2+}-CaM was completed to 99.2% of the backbone resonances. The chemical shifts of the ε-methyl protons in the methionines of Ca^{2+}-CaM in complex with the Munc13-1$^{458–492}$ peptide were compared with those of free Ca^{2+}-CaM. Most of the ε-methyl protons of the eight methionines in Ca^{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		
			D_1	D_2	D_3
(U-^{15}N, ^{13}C) CaM/Munc13-1$^{459–492}$ in 90% H_2O, 10% D_2O	^{1}H-^{15}N HSQC	900	2,048	128	–
	^{1}H-^{15}N NOESY-HSQC, t_{mix} 100 ms	900	2,048	48	128
	HNCO	700	2,048	46	46
	HN(CA)CO	600	1,024	60	60
	HNCACB	700	1,024	40	25
	CBCA(CO)NH	600	1,024	58	24
	H(CC)(CO)NH-TOCSY	700	2,048	30	64
	(H)CC(CO)NH-TOCSY	700	2,048	70	30
	^{1}H-^{13}C HSQC	600	2,048	128	–
(U-^{15}N, ^{13}C) CaM/Munc13-1$^{459–492}$ in 99% D_2O	^{1}H-^{13}C NOESY-HSQC t_{mix} 120 ms	700	2,048	48	58
	HCCH-TOCSY	600	2,048	128	64
	^{1}H-^{13}C HSQC	700	2,048	128	–
	^{1}H-^{13}C NOESY-HSQC (aromatic region) t_{mix} 130 ms	700	2,048	48	40
	HBCB(Caro)HG	600	2,048	46	–
	HBCB(Caro)HE	600	2,048	46	–
	^{1}H-^{13}C HMBC (methionines)	700	2,048	48	120
(U-^{15}N, ^{13}C) Munc13-1$^{458–492}$/CaM in 90% H_2O, 10% D_2O	^{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_{mix} 120 ms	600	2,048	180	114
	HNCO	900	1,024	64	32
	HN(CA)CO	600	1,024	48	46
	HNCACB	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

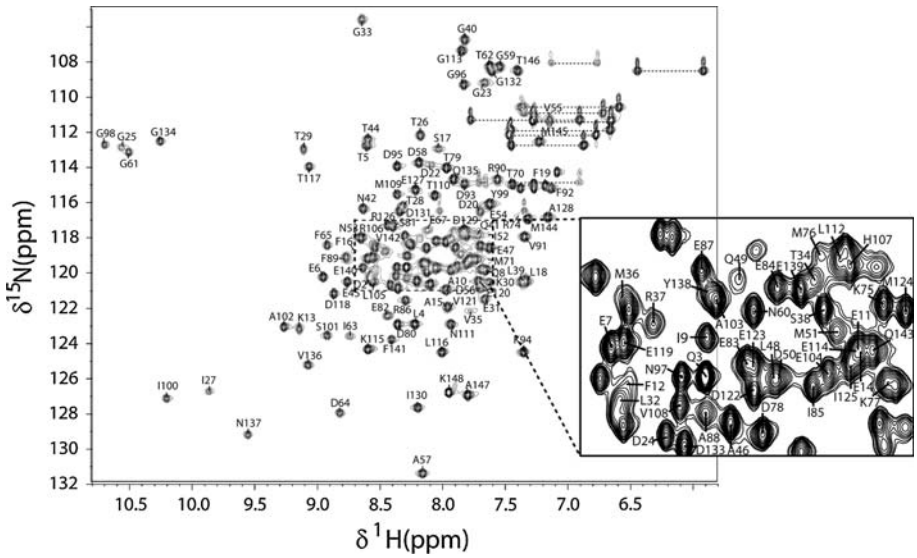

Fig. 1 ^{1}H-^{15}N-HSQC spectrum of the (U-^{15}N, ^{13}C) CaM/Munc13-1$^{459–492}$ complex measured at 35°C at 900 MHz. The highly overlapped central region is enlarged on the right side

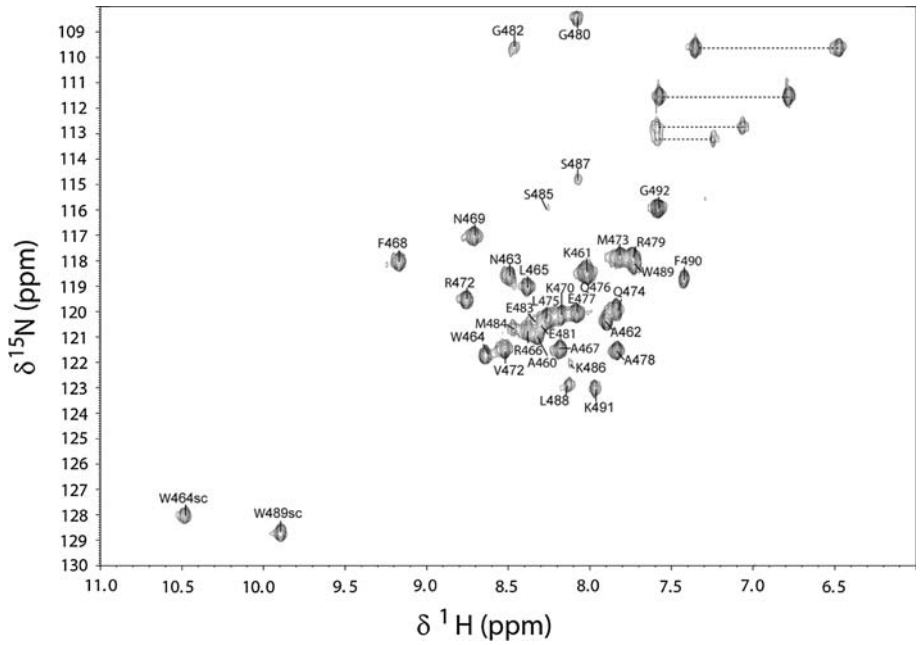

Fig. 2 ^{1}H-^{15}N-HSQC spectrum of the (U-^{15}N, ^{13}C) Munc13-$1^{458–492}$/CaM complex recorded at 35°C at 600 MHz

(−0.17 ppm), M124 (−1.17 ppm), M144 (−0.3 ppm) and M145 (−0.65 ppm). 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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