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NMR analysis of the structure of synaptobrevin and of its interaction with syntaxin

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Abstract

Synaptobrevin is a synaptic vesicle protein that has an essential role in exocytosis and forms the SNARE complex with syntaxin and SNAP-25. We have analyzed the structure of isolated synaptobrevin and its binary interaction with syntaxin using NMR spectroscopy. Our results demonstrate that isolated synaptobrevin is largely unfolded in solution. The entire SNARE motif of synaptobrevin is capable of interacting with the isolated C-terminal SNARE motif of syntaxin but only a few residues bind to the full-length cytoplasmic region of syntaxin. This result suggests an interaction between the N- and C-terminal regions of syntaxin that competes with core complex assembly.

Introduction

The release of neurotransmitters through synaptic vesicle exocytosis is regulated by a sequence of protein–protein interactions that are just beginning to be characterized in structural terms (reviewed in Rizo and Südhof, 1998). Central components of the exocytotic machinery are the synaptic vesicle protein synaptobrevin and the plasma membrane proteins syntaxin and SNAP-25, which are often referred to as SNAREs. Homologs of these three proteins are believed to be involved in all types of intracellular membrane traffic (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994). The three proteins form a highly stable complex known as the core or SNARE complex (Söllner et al., 1993) and assembly of this complex has been proposed to provide the driving force for membrane fusion (Südhof et al., 1993; Hanson et al., 1997; Lin and Scheller, 1997). The crystal structure of the core complex consists of a bundle of four long α-helices where one helix is from the C-terminal two-thirds of the cytoplasmic region of synaptobrevin, another helix is from the C-terminal domain of the cytoplasmic region of syntaxin, and the other two helices are from SNAP-25 (Sutton et al., 1998). In addition to the C-terminal SNARE motif, syntaxin contains an autonomously folded N-terminal domain that forms a three-helix bundle (Fernandez et al., 1998). While these structures have provided critical information to understand the function of the SNAREs, structural analyses of the SNARE motif sequences in isolation and in binary or ternary complexes are required to identify possible intermediates in core complex formation and to ultimately have a complete understanding of the molecular events that lead to membrane fusion. Analysis by circular dichroism has suggested that synaptobrevin is largely unstructured in isolation and only interacts weakly with the SNARE motif of syntaxin (Fasshauer et al., 1998), but no high resolution studies to corroborate these conclusions have been described. Here we have used NMR spectroscopy to characterize in detail the conformational behavior of isolated synaptobrevin and to study its interaction with syntaxin.

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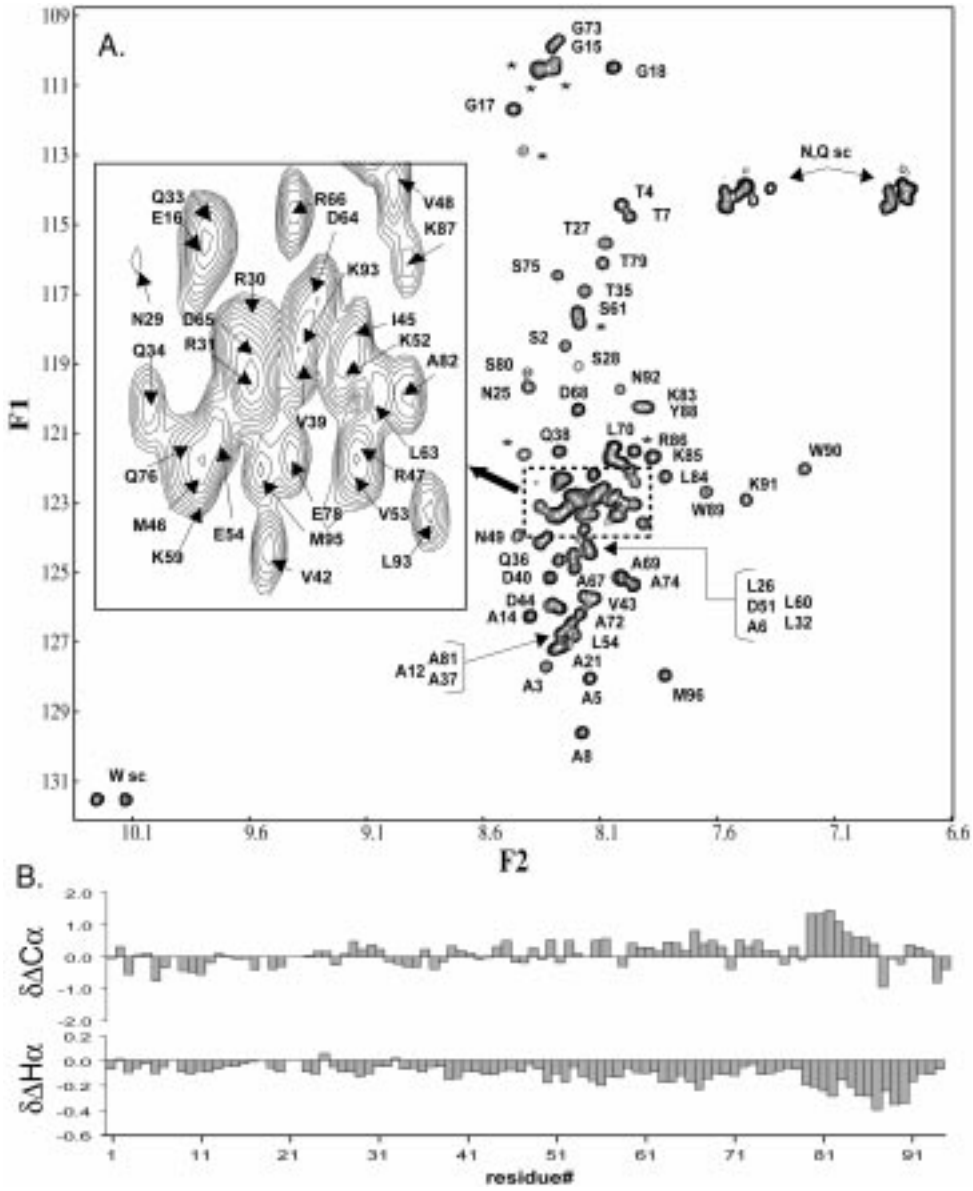

Figure 1. (A) ^{1}H-^{15}N HSQC spectrum of synaptobrevin-2 (1–96) illustrating the cross peak assignments. The inset shows an expansion of the most crowded region of the spectrum. A ∗ indicates cross peaks from an N-terminal sequence from the expression vector used, and sc refers to side chains. (B) Deviations of the Cα (ΔδCα) and Hα (ΔδHα) chemical shifts observed for synaptobrevin-2 (1–96) from the values expected for a random coil (in ppm), plotted as a function of the residue number. Random coil values were extracted from the BioMagResBank.

Materials and methods

The isolated cytoplasmic region of human synaptobrevin-2 (residues 1–96) was expressed as a GST-fusion protein in *Escherichia coli* BL21(DE3) cells, isolated by affinity chromatography on glutathione-sepharose (Pharmacia), cleaved from the fusion with thrombin, and purified by cation exchange chromatography on a MonoS column (Pharmacia). Uniform ^{15}N- and ^{13}C-labeling was achieved through expression in M9 minimal medium containing $^{15}NH_4Cl$ and $^{13}C_6$-glucose (Isotec) as the sole sources for nitrogen and carbon, respectively. The yield of pure protein was typically ca. 10 mg per liter of culture. NMR samples were prepared in 60 mM phosphate buffer (pH 6.1) using H_2O/D_2O (9:1) as the solvent. The protein concen-

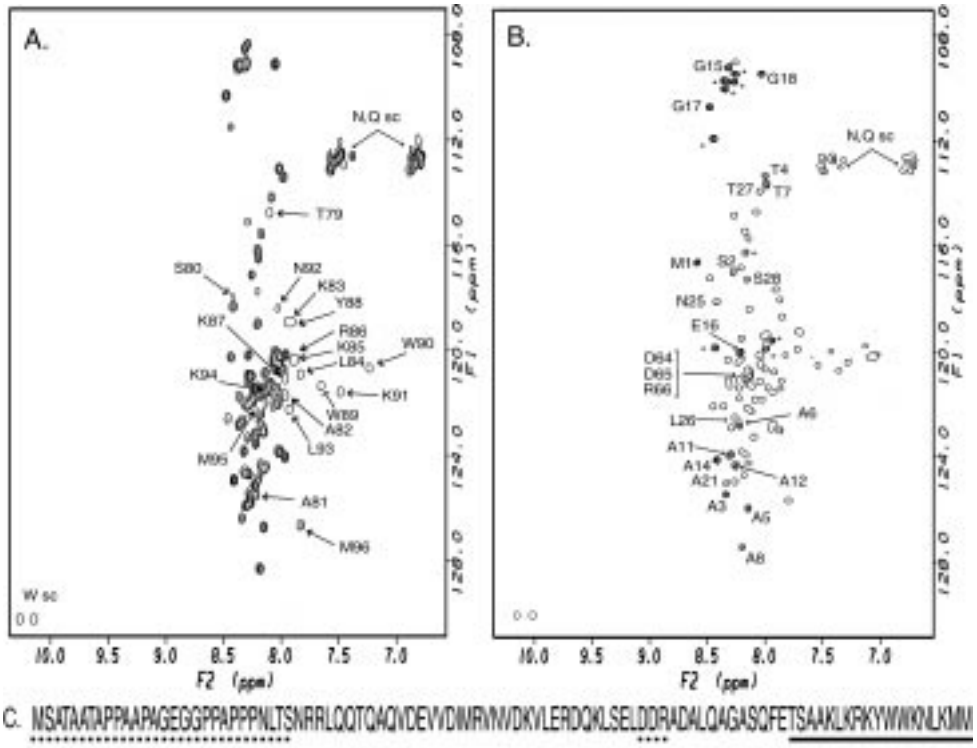

Figure 2. ^{1}H-^{15}N HSQC spectra of 80 μM synaptobrevin-2 (1–96) in the presence of 1 equiv of syntaxin 1A (1–264) (A) or syntaxin 1A (180–264) (B). The spectra are plotted with multiple contours and superimposed with ^{1}H-^{15}N HSQC spectra of isolated synaptobrevin-2 (1–96) (single contours). The spectra were acquired at pH 6.1 and 25 °C (A) or 5 °C (B). Cross peaks that disappear from the spectrum upon addition of syntaxin 1A (1–264) are labeled in (A) and indicated with a solid line below the sequence of synaptobrevin-2 in (C). Cross peaks that remain observable after addition of syntaxin 1A (180–264) are labeled in (B) and indicated by a dashed line in (C).

tration was 0.45 mM since synaptobrevin aggregates at higher concentrations. NMR experiments were acquired at 25 °C on a Varian Unity 500 spectrometer using a triple resonance probe. Sequential assignments were obtained from a series of 3D pulse field gradient-enhanced double and triple resonance experiments that incorporate water flip-back pulses and sensitivity enhancement in the ^{15}N-dimension whenever amide proton resonances are observed in the F3 dimension (Kay, 1993; Kay et al., 1994; Zhang et al., 1994). The spectral widths and number of complex points in the F3, F2 and F1 dimensions, with the number of scans per FID and the total measurement time indicated in parentheses, were: ^{1}H-^{15}N TOCSY-HSQC, 6800 × 926 × 4200 Hz, 512 × 40 × 128 (8 scans, 52 h); ^{1}H-^{15}N NOESY-HSQC, 6800 × 926 × 4200 Hz, 512 × 40 × 128 (8 scans, 55 h); HNCO, 7600 × 926 × 1620 Hz, 512 × 22 × 70 (8 scans, 15 h); HNCACB, 7600 × 926 × 7650 Hz, 512 × 22 × 34 (48 scans, 44 h); (H)C(CO)NH-TOCSY, 7600 × 926 × 7650 Hz, 512 × 22 × 50 (32 scans, 43 h); (H)CBCACO(CA)HA, 4000 × 1670 × 7650 Hz, 256 × 64 × 54 (16 scans, 61 h). The mixing times were 45 ms, 120 ms and 18 ms for the TOCSY-HSQC, NOESY-HSQC and (H)C(CO)NH-TOCSY experiments, respectively. Linear prediction was used to double the number of points in the F2 dimension of all spectra. After zero filling, Fourier transformation and removal of the aliphatic half of the F3 dimension for all spectra except (H)CBCACO(CA)HA, matrices of 512 × 128 × 256 points were obtained. All backbone ^{1}H-, ^{15}N- and ^{13}C-resonances were assigned except for those of one non-proline residue and those of four prolines that precede prolines in the sequence. Complete ^{1}H- and ^{13}C-resonance assignments (excluding aromatic and amide nuclei) were obtained for 65% of the side chains and partial assignments were obtained for the remaining side chains. The assignments have been deposited at the BioMagResBank under accession number 4272.

Results and discussion

The cross peak assignments of the ^{1}H-^{15}N HSQC spectrum of synaptobrevin-2 (1–96) are illustrated in Figure 1A. The small dispersion of amide proton chemical shifts is characteristic of unfolded proteins. In addition, the Hα and Cα chemical shifts exhibit only small deviations from random coil values (Figure 1B). The largest (negative) deviations for Hα protons are observed in a 15-residue segment at the C-terminus, suggesting that this region may populate α-helical conformations a substantial amount of time. Correspondingly, positive deviations of Cα chemical shifts are observed in this segment, although a few null or even negative deviations are observed around residues Tyr^{88}-Trp^{89}-Trp^{90} that may arise from the abundance of aromatic side chains. In the 3D ^{1}H-^{15}N NOESY-HSQC spectrum, only intraresidue and sequential NOEs were observed throughout the sequence, and the few $d_{NN}(i,i+1)$ NOEs observed were mostly concentrated in the C-terminal 15-residue segment (data not shown). All these observations demonstrate that the isolated cytoplasmic region of synaptobrevin-2 is basically unfolded in solution and contains nascent helical structure at the C-terminus. A small amount of nascent helical structure may also exist in other regions of the molecule, as suggested by slight but systematic upfield shifts of Hα protons with respect to random coil values, which often coincide with down field shifts in Cα carbons (Figure 1B).

To study the interaction of synaptobrevin with syntaxin, we prepared unlabeled fragments corresponding to the entire cytoplasmic region of syntaxin 1A (residues 1–264) and to the C-terminal SNARE motif (residues 180–264) as described (Shao et al., 1997). The effects of addition of these fragments on the ^{1}H-^{15}N HSQC spectrum of ^{15}N-labeled synaptobrevin-2 (1–96) were then analyzed. Most of the spectrum remained unchanged upon addition of syntaxin 1A (1–264) but the cross peaks from the C-terminal 18 residues were severely broadened, most of them beyond detection (Figure 2A). The severe broadening reflects binding to the large syntaxin fragment (31 kDa) while cross peaks from the rest of synaptobrevin remain sharp because of internal motions associated with their unfolded nature. ^{1}H-^{15}N HSQC spectra of synaptobrevin-2 (1–96) in the presence of the syntaxin 1A (180–264) fragment were acquired at 5 °C to avoid protein precipitation. The spectra were compared with a ^{1}H-^{15}N HSQC spectrum of synaptobrevin-2 (1–96) acquired also at 5 °C. Cross peak assignments at this temperature were deduced from those at 25 °C by performing a temperature titration monitored by ^{1}H-^{15}N HSQC spectra. In the presence of syntaxin 1A (180–264), most cross peaks of synaptobrevin-2 (1–96) disappeared from the spectrum and only those from the N-terminal proline-rich region and a short stretch of 3 residues remained sharp (Figure 2B). While the expected molecular weight of a binary complex of synaptobrevin-2 (1–96) with syntaxin 1A (180–264) is moderately large (22 kDa), the absence of new cross peaks corresponding to the bound region of synaptobrevin can be attributed to the low temperature used and perhaps to the formation of higher order complexes. The bound region of synaptobrevin-2 (residues ca. 25 to 96) coincides with the region that participates in core complex formation (Sutton et al., 1998), showing that the entire SNARE motif of synaptobrevin-2 is able to interact with the SNARE motif of syntaxin 1A. However, only a short C-terminal region of synaptobrevin-2 is able to bind to the full-length cytoplasmic region of syntaxin 1A, which may be aided by the intrinsic tendency of this region to form α-helix (see above). These results provide a direct demonstration that the N-terminal region of syntaxin prevents full binding between the SNARE motifs of syntaxin and synaptobrevin. Such inhibition had been previously suggested from biochemical analysis of interactions between syntaxin and synaptobrevin but the observation or not of inhibition depended on the assay used (Calakos et al., 1994).

Conclusions

Our results have important implications to develop models of the mechanism of synaptic vesicle exocytosis. Core complex formation is widely believed to be a required step for exocytosis. The structural properties of synaptobrevin described here appear to be shared by the SNARE motifs of SNAP-25 and syntaxin since ^{1}H-^{15}N HSQC spectra of SNAP-25 and the isolated C-terminal region of syntaxin also exhibit narrow dispersion of ^{1}H chemical shifts (our unpublished results). These observations, and the fact that neurotransmitter release occurs extremely fast after Ca^{2+} influx, suggest that the α-helical structure of synaptobrevin (and that of the other SNARE motifs) may be preformed before Ca^{2+} influx through partial formation of the core complex or through binding to other protein(s). The resonance assignments described here will facilitate future studies of the binding interactions

involving synaptobrevin. Our results also show that the SNARE motifs of synaptobrevin and syntaxin are capable of fully interacting in the absence of SNAP-25 but full binding is prevented by the N-terminal region of syntaxin. This observation suggests that in isolated syntaxin the N-terminal and C-terminal regions interact. Studies of the yeast syntaxin homolog SSO1p have also indicated an N- to C-terminal interaction, which was proposed to regulate assembly of the yeast SNARE complex (Nicholson et al., 1998; Fiebig et al., 1999). This similarity between the neuronal and yeast systems adds to the evidence supporting the universality of the mechanism of intracellular membrane fusion.

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