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# Letter to the Editor: $^{1}$H, $^{13}$C, and $^{15}$N assignments of un-myristoylated $Ca^{2+}$-frequenin, a synaptic efficacy modulator

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## Biological context

Frequenin is a 190 amino acid N-terminally myristoylated globular protein involved in the efficient transduction of nerve stimuli in synaptic nerve endings where it potentiates neurotransmitter release. It belongs to a large family of neuronal calcium binding proteins, which share a common structural motif, the EF-hand, and of which two sub-groups with different calcium binding characteristics exist. Frequenin was originally isolated from *Drosophila* based on its ability to potentiate post-synaptic efficacy and to facilitate neurotransmitter release at neuromuscular junctions (Pongs et al., 1993). In yeast, frequenin has been shown to activate phosphotiylinositol-4-OH kinase, thus implicating it as an important regulator of phosphoinositides, membrane-specific second messengers which control functions such as membrane trafficking, cytoskeletal organisation and other signalling proteins (Hendricks et al., 1999). Frequenin has also been observed to stimulate the activity of calcineurin, phosphodiesterase, NO-synthetase and of guanylate cyclase (Pongs et al., 1993). The assignments reported here provide the basis for a structural investigation of the function and calcium binding properties of frequenin.

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## Methods and results

Recombinant un-myristoylated rat frequenin (rfrq) was expressed using the pET-16b vector in *E. coli* strain BL21(DE3). These were grown at 37 °C in Martek9 medium (Martek Bioscience Corp., MD) and induced with IPTG at an $A_{600}$ of 0.5. The overnight growth was pelleted, resuspended in 15 ml of 100 mM KCl/1 mM EGTA/1 mM DTT/1 mM $MgCl_2$/50 mM potassium HEPES, pH 7.5 and disrupted by sonication. Cell debris, insoluble proteins and nucleic acids were precipitated with streptomycin sulfate. $CaCl_2$ was added to a final concentration of 2 mM and after filtration the lysate was applied to a Phenyl Sepharose High Performance column (Pharmacia Biotech, Sweden), pre-equilibrated with buffer A containing 1 mM $MgCl_2$/1 mM DTT/1 mM $CaCl_2$/20 mM Tris-HCl, pH 7.9. Rfrq was eluted with 20–25 ml of buffer B containing 1 mM $MgCl_2$/1 mM DTT/2 mM EGTA/20 mM Tris-HCl, pH 7.9. Calcium was added to a final concentration of 4 mM. The sample was applied to a HiTrap Q column (Pharmacia Biotech, Sweden) equilibrated with buffer A, washed and eluted with a linear NaCl gradient in buffer A.

Uniformly $^{15}$N-, $^{13}$C- and $^{15}$N/$^{13}$C-labeled proteins were obtained by growing the over-expressing bacteria in the respective Martek9 media. Purity and a labelling efficiency of >97% were determined using ESI-MS. No N-terminal myristoylation was present and the initiating Met was processed in the proteins. The protein concentration was 1.4 mM in all cases and the pH 7.2, 8 mM $CaCl_2$, 14 mM DTT. All spectra were recorded at 37 °C on a Varian UNITY*plus* 600 MHz spectrometer.

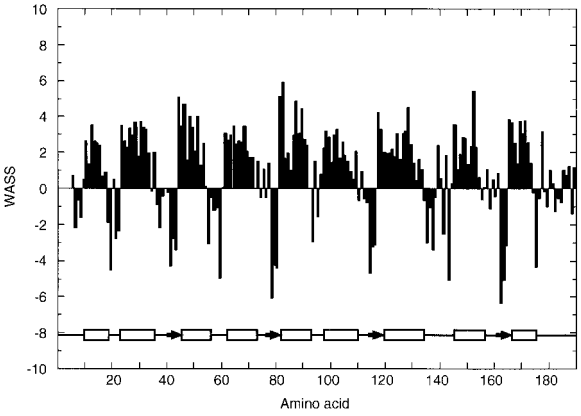


*Figure 1.* The Weighted Average Secondary chemical Shifts (WASS) of $^{1}H^{\alpha}$, $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and $^{13}C'$ give information on the secondary structure of rfrq. For each residue, the deviation of the chemical shift values of four atoms, $^{1}H^{\alpha}$, $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and $^{13}C'$, from their random coil values ($\delta_{\text{random coil}}$ (ppm)-$\delta_{\text{r}-\text{frq}}$ (ppm)) are evaluated and normalised to the reported random coil range for each residue (Wishart, 1992). These four normalised values are summed and averaged for each residue. Thus the secondary structure of each residue can be evaluated using one common value instead of four. Helices are assigned from four or more consecutive positive values and β-structures from three or more consecutive negative values.

The following NMR spectra were recorded and used for assignments: 2D-$^{15}$N,$^{1}$H-HSQC, 3D-$^{15}$N-TOCSY-HSQC, 3D-$^{15}$N-NOESY-HSQC, 3D-$^{13}$C-NOESY-HSQC, 3D-HNCA, 3D-HNCO, 3D HN(CO)-CA, 3D CBCA(CO)HN, 3D CBCACOHA, 3D HCCH-TOCSY, 4D-$^{13}$C-NOESY-HSQC (Bax and Grzesiek, 1993; Vuister et al., 1993; Kay et al., 1994; Yamazaki et al., 1994; Zhang et al., 1994; Cavanagh et al., 1996). All spectra were processed using MNMR (Kjær et al., 1994). $^{1}$H chemical shifts were referenced to the methyl proton resonances of an external DSS standard at 0.00 ppm, and $^{15}$N and $^{13}$C chemical shifts were indirectly calibrated. The data were analysed using PRONTO (Kjær et al., 1994).

## Extent of assignments and data deposition

The sequential assignment procedure was heavily based on $^{15}$N-correlations due to their excellent dispersion. The first five residues were not observed in the spectra. Complete backbone assignments of $^{1}$H, $^{13}$C and $^{15}$N resonances have been obtained for all but 10 of the 190 residues, of which seven are prolines. Full assignment of all side chain resonances, disregarding -OH's and -$NH_2$'s, was obtained for 101 residues, and the majority of the resonances in the remaining residues were assigned, except for a few lysines and arginines. Overlap problems occurred especially in the central part of the $^{15}$N-edited spectra, in the $^{13}C^{\alpha}$-region, and in the aromatic region. The latter hindered full assignment of Trp, Tyr and Phe side chains. Nine α-helices were identified from the pattern of short- and medium-range NOEs and from the weighted average secondary shifts of backbone atoms $^{13}C'$, $^{13}C^{\alpha}$, $^{13}C^{\beta}$, and $^{15}$N of each residue, Figure 1. There are four three-residue β-strand structures with consecutive large secondary shifts, located in the typical calcium binding EF-hand loops.

The assignments of $^{1}$H, $^{13}$C and $^{15}$N chemical shifts of $Ca^{2+}$-loaded un-myristoylated rfrq have been deposited in the BioMagResBank database (accession number 4378).

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