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Letter to the Editor: Sequence specific $^{1}H^{N}$, ^{13}C and ^{15}N resonance assignments of a novel calcium-binding protein from *Entamoeba histolytica*

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Biological context

EF-hand calcium-binding proteins play a key role in various cellular processes, including the pathogenesis exhibited by *Entamoeba histolytica*, the causative agent of amoebiasis (Ravdinet al., 1985; Munoz et al., 1992). The precise mechanism involving calcium, calcium-binding proteins and the role of various Ca^{+2} sensitive pathways in host–parasite interactions is still poorly understood. We have been involved in the study of the structure–function relationship of two of the three calcium-binding proteins identified, isolated and sequenced till date in *Entamoeba histolytica* (abbreviated as *Eh*CaBP1 and *Eh*CaBP2) (Prasad et al., 1992; Sahu et al., 1999a, b; Atreya et al., 2001; Chakrabarty et al., 2004).

Similar to calmodulin (CaM), with which it shares a 29% sequence homology, *Eh*CaBP1 has two independent globular domains connected by a flexible linker, each containing a pair of EF hands. However, *Eh*CaBP1 was found to be structurally and functionally distinct from CaM. *Eh*CaBP1 has a more open conformation of the Ca^{+2} binding loops in the C-terminal domain with larger water-exposed total hydrophobic surface area as compared to CaM and Troponin C (Atreya et al., 2001). In addition, the linker region was found to be more flexible as compared to that of CaM.

*Eh*CaBP2, which is also 134 amino-acid residues long, is an isoform of *Eh*CaBP1. Both, *Eh*CaBP1 and *Eh*CaBP2, are encoded by non-allelic genes of the same size (402). The two genes showed 79% identity which dropped to 40% in the 75-nucleotide linker region. Based on the genetic sequence, the two proteins are expected to have differences in the central linker region, but not in the calcium-binding domains. These observations were supported by the differences in their binding ability to variable sets of proteins and peptides (Chakrabarty et al., 2004; Sahoo et al., 2004). In addition, they showed uniqueness in activating endogenous kinases and in the calcium concentration required for their optimal activity. These preliminary results suggest that the two proteins are involved in different calcium signal transduction pathways. Presence of multiple CaM-like genes with divergent functions and non-coding sequences in *E. histolytica* is similar to the identification of CaM gene families in plants (Lee et al., 1995). Different CaMs are thought to be involved in slightly diverse signal transduction processes initiated by different signals.

In order to understand these protein functions through a better understanding of their structures, we have initiated the 3D structure determination of the calcium bound form of *Eh*CaBP2 using NMR.

Methods and results

The gene encoding *Eh*CaBP2 was cloned in pET30a vector for expression in *E. coli* BL21 (DE3) cells. In our present study, it was observed that after a couple of days from its isolation the purified *Eh*CaBP2, became unstable at pH values above 5.5. This is in contrast to most other known calcium-binding proteins. This unusual pH dependence and stability of *Eh*CaBP2 is currently under investigation in our laboratory. Below its isoelectric pH, the aqueous solution of *Eh*CaBP2 maintained its isotropic stable form for many months. Hence, the procedure for expression and purification of *Eh*CaBP2 has been slightly modified from that described earlier (Chakrabarty et al., 2004).

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1 g $^{15}NH_4Cl$ and 2.5 g ^{13}C-glucose per litre of the M9 media were used as the sole sources of ^{15}N and ^{13}C, respectively, for the production of isotopically ^{15}N-labeled and doubly-labeled (^{15}N and ^{13}C) protein, *Eh*CaBP2. The bacterial cells in their mid-log phase ($OD_{600\ nm} = 0.6$) were treated with IPTG for 4–5 h. These induced bacterial cells were then suspended in 25 mM Tris-HCl, pH 7.5, containing 2 mM EGTA followed by ultrasonication. The clear supernatant containing recombinant *Eh*CaBP2, was purified using DEAE-52 anionic exchanger (Chakrabarty et al., 2004). Immediately after collecting the purified protein fractions from the chromatographic column, the pH of the pooled protein solution was lowered to 4.0–4.2 using 3 M acetate buffer. For further purification, the protein solution was subjected to heat treatment in a boiling water bath for 3 min followed by separation of the heat precipitable proteins. The supernatant was finally subjected to ultrafiltration using a 3 kDa cut-off membrane for achieving NMR concentrations. Approximately 55 mg of purified labeled *Eh*CaBP2 was produced from 1 l of the bacterial culture, the purity of which was confirmed by SDS-PAGE analysis.

NMR experiments were conducted on a Varian Unity+ 600 MHz NMR spectrometer equipped with a pulsed-field-gradient unit and a triple resonance probe with actively shielded Z-gradients, operating at a ^{1}H frequency of 600.051 MHz. All NMR measurements were performed at 35 °C using a 1.5 mM, 0.6 ml solution of *Eh*CaBP2 in a mixed solvent of 90% H_2O and 10% $^{2}H_2O$, containing 10 mM $CaCl_2$. The pH of the protein sample was maintained between 4.0–4.2, with the total buffer concentration being maintained at 100–150 mM. The parameters used in recording and processing various multi-dimensional NMR experiments with [u–^{15}N/^{13}C]-labeled *Eh*CaBP2 are compiled in a supplementary table. Data processing was done on Silicon Graphics Octane workstation using the FELIX 2002 software (Biosym Technologies, San Diego, CA, USA).

Proton chemical shifts were calibrated relative to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 35 °C (0 ppm). Carbon and nitrogen chemical shifts were calibrated indirectly from DSS.

Extent of assignments and data deposition

*Eh*CaBP2 is free of Cys, His and Trp residues. Sequence-specific resonance assignments (Wüthrich, 1986) for nearly all of $^{1}H^{N}$, ^{15}N, $^{13}C^{\alpha}$, $^{13}C^{\beta}$ and $^{13}C'$ spins have been carried out with the concerted use of HNCA (Grzesiek et al., 1992), HN(CA)CO (Clubb et al., 1992), HNCO (Kay et al., 1990), CBCANH (Wittekind et al., 1993) and CBCA(CO)NH (Kay et al., 1993) spectra. It was not possible to unambiguously assign the resonances for M1, A2, E3, K60, E67, and Q68. In addition, the ^{15}N and ^{1}H spins of A4, K7, F61, and E73 and the $^{13}C^{\beta}$ resonance of V65 could not be assigned. Most of the unassigned amino acids either lie at the N-terminal end of the polypeptide chain or belong to the flexible linker region, joining the two globular domains. These initial results suggest a more flexible conformation for *Eh*CaBP2 as compared to *Eh*CaBP1. The chemical shifts of all resonances thus obtained have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under Accession Number 6433.

Supplementary material to this paper is available in electronic format at http://dx.doi.org/10.1007/s10858-005-2471-z.

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