CHAPTER 2

Expression, Purification, Crystallization and Preliminary Crystallographic Analysis of EhCaBP2 and its Complexes

2.1 Introduction

Entamoeba histolytica is the etiological agent for human amoebic colitis and liver abscess and causes a high level of morbidity and mortality worldwide, particularly in developing countries. There are a number of studies that show the involvement of Ca^{2+} and its binding proteins in amoebic pathogenesis (Ravdin et al., 1988). Previously, a novel Ca^{2+}-binding protein from *E. histolytica* (EhCaBP1) has been characterized and its three dimensional structure has been derived using multidimensional nuclear magnetic resonance (NMR) spectroscopic techniques in the apo form as well as in a complexed form with Ca^{2+} (Atreya et al., 2001; Yadava et al., 1997). EhCaBP1 is a 14.7 kDa (134 amino-acid residues) protein that has been shown to participate in cytoskeletal dynamics (Sahoo et al., 2004). The study reveals the presence of two globular domains connected by a flexible linker region spanning eight amino-acid residues. EhCaBP1 binds to four Ca^{2+} ions with high affinity (two in each domain) and it is structurally related to calmodulin (CaM) and troponin C (TnC), despite having low sequence homology with these proteins. The NMR structure shows a more open C- terminal domain for EhCaBP1 with a larger water-exposed total hydrophobic surface area compared with CaM and TnC (Atreya et al., 2001). Further dissimilarities between the structures include the presence of two Gly residues (Gly 63 and Gly 67) in the central linker region in EhCaBP1, which seem to impart a greater flexibility compared with CaM and TnC and may also play a crucial role in its biological function. The major differences in the structure of EhCaBP1 with respect to those of CaM and TnC are in the Ca^{2+} binding loops, interhelical angles and exposed hydrophobic surface. These structural features make EhCaBP1 functionally distinct from other CaM like Ca^{2+} binding proteins. In a recent study, a paralogous isoform of EhCaBP1, EhCaBP2, was identified and partially characterized (Chakrabarty

et al., 2004). The two isoforms are encoded by genes of the same size (402 bp). Comparison between the two genes showed an overall identity of 79 % at the nucleotide-sequence level (78 % at the protein level). This identity dropped to 40 % in the 75-nucleotide central region (56 % at the protein level) between the second and third Ca^{2+} binding domains. An array of biochemical studies indicated that despite their structural similarities, the two EhCaBPs are functionally distinct.

EhCaBP2, a CaM-related protein involved in cytoskeletal dynamics, may bind to the IQ motifs of myosin. Myosins, neuronal growth proteins, voltage-gated channels and certain signaling molecules contain IQ motifs (Bahler & Rhoads, 2002) that can bind to either CaM or CaM-related proteins. These motifs are of about ~25 amino acids in length and conform to the consensus sequence (I,L,V)QxxxRxxxx(R,K) (reviewed by Bahler & Rhoads, 2002).

2.2 Materials and Methods

2.2.1 Sources of Materials

E. coli strain DH5α was obtained from Bethesda Research Labs (B.R.L., U.S.A). DEAE & Phenyl Sepharose resin was purchased from Amersham Pharmacia (U.S.A.), Promega (U.S.A.), Sigma (U.S.A.) and other reagents from Sigma Aldrich (U.S.A.) and Qualigens (India). *E. coli* media components were from DIFCO (U.S.A.). All concentrations indicated in percentage are in (w/v) basis unless stated otherwise. All solutions were prepared in double distilled water unless stated otherwise. Autoclaving was done at a pressure of 15 lbs per square inch for 20 min).

2.2.2 Organisms and Growth Conditions

E. coli DH5-α has the genotype: *SupE*44 *lac*U169 (ϕ80 *lacZ* M15) *hsdR17 recA1 endA1 gyrA*96 *thi*-1 *relA*1. Cells from an agar stab or frozen glycerol stock was first streaked on an LB plate (containing the appropriate antibiotic wherever necessary) and allowed to grow overnight at 37°C. Liquid cultures in LB medium were initiated from a single colony and were grown with constant shaking at 225 rpm at 37°C. The cells were grown overnight, were used as inoculum for further growth by diluting 100 fold in fresh LB medium and grown with aeration at 37°C for 3-4 h to obtain log phase cultures.

2.2.3 Culture Media

2.2.3.1 Luria Broth (LB)

Bacterial cells were grown in Luria Broth (LB). It was prepared by dissolving 25 gms of LB powder (Amersham) in 1 liter of distilled water and pH adjusted to 7.0 using 2 N NaOH. The medium was sterilized by autoclaving.

2.2.3.2 LB Agar

LB agar was prepared by adding 1.5 % (w/v) of Bacto-Agar to LB medium and sterilized by autoclaving. Ampicillin was added to a final concentration of 100 µg/ml (when required) after cooling the LB agar to around 55°C and plates were poured.

2.2.4 Plasmid DNA Isolation from *E. coli* Transformants

2.2.4.1 Preparation of competent cells and transformation (Hanahan, 1983)

Single colony of *E. coli* [strain DH5α, BL21(DE3), SG130069] was grown overnight in 5ml LB medium and 1% inoculum was added to 50 ml LB medium in 500 ml flask. The cells were grown at 37°C to an OD_{600} of 0.38-0.42. The cells were vigorously shaken on ice water for 15 min and were thereafter collected by centrifugation at 5000 rpm for 5 min at 4°C and resuspended in 25 ml ice-cold filter sterilized 0.1 M $CaCl_2$. Cells were incubated for 1 h on ice with occasional shaking; the cells were collected again by centrifugation at 5000 rpm for 5 min at 4°C. The halo shaped pellet was finally resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$. The competent cells were stored in 15% glycerol stocks in 100 µl aliquots at -70°C.

Competent cells were thawed on ice and to 100 µl cell suspension, 5-10 ng of plasmid DNA was added. The cells were incubated on ice for 30 min. Cells were then given a heat shock at 42°C for 90 s and incubated on ice for 5 min. 0.9 ml of LB was added to the cells and the cells were grown at 37°C for 1 h at 200 rpm. Transformants were plated on LB agar plates with appropriate antibiotic and incubated at 37°C for 14-16 h.

2.2.4.2 Mini-preparation of plasmid DNA (Alkaline lysis method) (Birnboim and Doly, 1979)

A single colony harboring the desired plasmid was inoculated in 2 ml of LB medium containing appropriate antibiotic and grown overnight at 37°C. The cells were pelleted at 6,000 rpm for 5 min and soup was aspirated out. The pellet was suspended in 100 µl of solution I (50 mM glucose, 25 mM Tris-Cl pH 7.5, 10 mM EDTA pH 8.0). To the tube

200 µl of freshly prepared solution II (0.2 N NaOH and 1% SDS) was added, mixed gently by inverting and incubated on ice for 5 min, 150 µl of chilled solution III (3 M potassium acetate, pH 5.2) was then added and the contents were mixed gently by inverting the tube and kept on ice for 10 min. The mixture was centrifuged at 14,000 rpm for 10 min at 4°C. The soup was transferred to the fresh tube and 0.7 volumes of isopropanol was added and centrifuged at 14000 rpm for 10 min. The pellet was washed with 70 % ethanol by centrifugation at 14000 rpm for 5 min at RT. The soup was discarded and pellet was air dried. The dried pellet was suspended in $T_{10}E_1$-RNAse or autoclaved Milli-Q.

2.2.4.3 Agarose gel electrophoresis

The agarose concentrations used in electrophoresis separation were chosen based on the size of the DNA to be resolved. Agarose was melted in 0.5X TBE [45 mM Tris- borate and 1mM EDTA, pH 8.0] by heating and was cooled to about 50^{0}C before adding 0.5 µg/ml of ethidium bromide. The molten agarose was poured in a tray and allowed to gel. After the gel had set, DNA samples were loaded and electrophoresed in 0.5X to 1X TBE in appropriate electric field strength for optimum separation. The DNA was visualized at 302 nm using a UV trans-illuminator.

2.2.5 SDS-Polyacrylamide gel electrophoresis (Laemmli, 1970)

SDS-PAGE was carried out under reducing conditions. The separating gels (10-14 % arylamide as per need) was prepared using acrylamide (acrylamide:bis-acrylamide =29:1) in 1.5 % Tris-Cl pH 8.8, 0.1 % (w/v) SDS, 0.04 % (w/v) APS and TEMED. After polymerization of separating gel, stacking gel was poured. The stacking gel contained 4 % acrylamide in 0.5 % Tris-Cl pH 6.8, 0.1 % (w/v) SDS, 0.04 % (w/v) APS and TEMED. Prewarmed samples and 4X SDS-PAGE loading bye [125 mM Tris-Cl pH 6.8, 4 % (w/v) SDS, 10 % (w/v) 2-mercaptoethanol, 20 % (v/v) glycerol and 0.2 % (w/v) bromophenol blue] were mixed to 1X dye concentration and reboiled for 2 min. After electrophoresis, proteins were fixed in the gel by incubating in fixing solution (50 %

methanol, 7.5 % acetic acid) and detected by Coomassie Brilliant Blue (0.25 % CBB R-250 in fixing solution) staining for 1 h. The gels were destained in the fixing solution and dried.

2.2.6 Protein estimation (BCA assay)

The amount of protein in a sample was estimated by the bicinchoninic acid assay using BSA as the standard (Smith et al, 1985). The working solution was prepared by mixing bicinchoninic acid (Sigma, U.S.A.) and 4 % copper sulphate in a ratio of 50:1. To 20 µl of protein (appropriate dilutions) was added 180 µl of the working solution in a microtitre plate and incubated at 37°C for 30 min. The absorbance was taken at 562 nm using a microtiter plate reader (Bio-Rad, U.S.A.).

2.2.7 Expression and purification of recombinant EhCaBP2 in *Escherichia coli.*

A 5ml culture (LB + 60 µg/ml kanamycin) of a single well-isolated EhCaBP2 colony was inoculated in a 100 ml conical flask (Borosil) and incubated overnight at 37^{0}C at 220 rpm. A 200 ml culture (LB + 60 µg/ml kanamycin) in 1L of conical flask was inoculated with 1% of the above culture and grown at 37^{0}C/220 rpm till the OD_{600} reaches between 0.5 to 0.7. The bacterial culture was induced with 1mM IPTG and incubated for 3-4 hr at 37°C/220 rpm. The induced bacteria was collected at 6000 rpm at 4^{0}C for 10 min and washed once with Wash buffer (50 mM Tris.Cl, pH 7.5 and 100 mM NaCl). The cell pellet was suspended in 1/25th volume of the original culture in 50 mM Tris.Cl pH 7.5 and 2 mM EGTA). The cells were lysed by freeze-thawing thrice in liquid nitrogen followed by sonication (3 X 30sec, full burst, with 1min interval) on ice. The sonicated sample were spun down at 12,000 X g for 30 min at 4^{0}C and the supernatant was loaded on to a packed DEAE Sepharose column.

The purification of EhCaBP2 was performed on DEAE anion exchange chromatography. The column was equilibrated with 20 bed-volumes of 50 mM of Tris-Cl (pH 7.5) + 2 mM

EGTA (pH 7.5). The sample was loaded to the column and the flow through was collected. The column was washed with 20 bed-volumes of 50 mM of Tris-Cl (pH 7.5), 2 mM EGTA (pH 7.5). The protein was eluted in 1ml fractions with 50 mM Tris-Cl (pH 7.5), 10 mM $CaCl_2$ and the OD_{280} was taken. This protein was further purified using Phenyl Sepharose affinity chromatography. The protein was dialyzed in 5 mM $CaCl_2$, 50 mM HEPES buffer pH 7.5 and passed through the Phenyl Sepharose column, which had been pre-equilibrated with the same buffer. This column was washed with 500 mM NaCl with 50 mM HEPES buffer pH 7.5, 5 mM $CaCl_2$ to remove non specific bound proteins. The column was further washed with 5 mM $CaCl_2$, 50 mM Hepes buffer pH 7.5. The EhCaBP2 was eluted with 5 mM EGTA, 10 mM EDTA in 25 mM cacodylate buffer pH 5.0. The sample was 99% pure as estimated by SDS–PAGE [fig. (2.3.1)]. The CaBP2 was concentrated to 15 mg/ml [estimated by BCA assay (Smith et al., 1985)] using Centricon microconcentration devices (Amicon Inc. Beverly, MA, USA) and used for crystallization.

2.2.8 Preparation of Sr–EhCaBP2 complex

The purified EhCaBP2 was dialyzed against 6 M urea, 10 mM EDTA to remove the bound Ca^{2+} from the EhCaBP2. Subsequently, this apo-form EhCaBP2 was dialyzed against 10 mM cacodylate buffer with 5 mM $SrCl_2$ with two buffer changes to form the Sr–EhCaBP2 complex. The Sr–EhCaBP2 complex was concentrated to 15 mg/ml [estimated by BCA assay (Smith et al., 1985)] using Centricon microconcentration devices (Amicon Inc. Beverly, MA, USA) and used for crystallization.

2.2.9 Preparation of IQ1–EhCaBP2 complex

EhCaBP2 was mixed with IQ1 peptide motif (generously donated by R. Dominguez, Boston Biomedical Research Institute, Boston, MA, USA) in a 1:2 molar ratio. Initially, the IQ1 peptide was dissolved in DMSO and then added to EhCaBP2 which had been pre equilibrated with 50 mM acetate buffer pH 3.5. This mixture of IQ1 and EhCaBP2 was dialyzed against 50 mM cacodylate pH 5.0, 5 mM $CaCl_2$. When a similar experiment was performed without calcium, more precipitate was observed in the dialysis membrane.

However, when the pH was raised to 5.0 some precipitate was observed in the dialysis membrane. IQ1 was bound to EhCaBP2 and the excess IQ1 was precipitated in the presence of calcium, while all the IQ1 precipitated and none was bound to EhCaBP2 in the absence of calcium. It is noted that EhCaBP2 activates kinase in a calcium-dependent manner (Chakrabarty et al., 2004). The IQ1–EhCaBP2 complex was concentrated to 15 mg/ml [estimated by BCA assay (Smith et al., 1985)] using Centricon microconcentration devices (Amicon Inc. Beverly, MA, USA) and used for crystallization.

2.2.10 Crystallization

Initial crystallization experiments were performed at both 289 K and cold-room (277 K) temperature for EhCaBP2 and its complex with IQ1 using the hanging-drop vapor-diffusion method. The crystallization trials were performed using different ratios of protein and precipitant solution (1:1, 1.5:1, 2:1, and 3:1) and then equilibrated by vapor diffusion with same precipitant. The details of crystallization conditions for EhCaBP2 and its complexes are summarized in Table 2.3.3.

2.2.11 Data collection and processing

The X-ray diffraction experiments were performed at 100 K using crystals mounted in cryoloops at cryogenic conditions and flash frozen in liquid nitrogen. All data sets were collected at 100 K. The X-ray diffraction data from EhCaBP2 crystals were collected at BNL (Brookhaven National Laboratory) using beam line X9. The data from Sr–EhCaBP2 crystals were collected at CHESS using the A1 beam line. The data from IQ1–EhCaBP2 crystals were collected at SSRL beam line 11-1. All the data sets were indexed and scaled with the programs DENZO and SCALEPACK (Otwinowski & Minor, 1997).

2.3 Results and Discussion

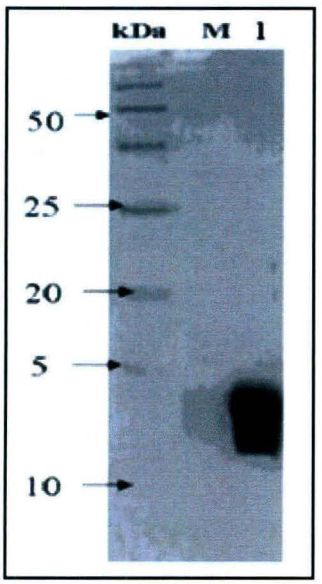


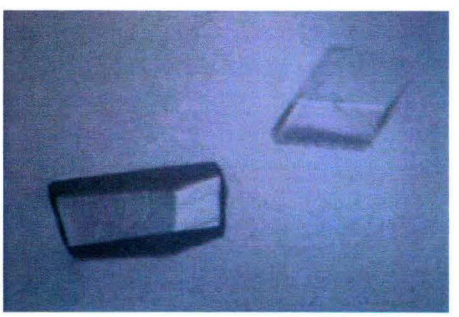
Table 2.3.3

Crystallization of EhCaBP2 and its complexes.

EhCaBP2	45–60 % (w/v) MPD, 5 mM $CaCl_2$, 50 mM acetate buffer , pH 4.6
St–EhCaBP2	40–50 % (w/v) MPD, 50 mM acetate buffer pH 4.6, 5 mM $SrCl_2$
IQ1–EhCaBP2	30 % (w/v) ethanol, 40–50 % (w/v) MPD, 50 mM cacodylate buffer, pH 6.5

Fig. 2.3.1 14 % SDS PAGE showing purified EhCaBP2 in lane 1.

(a)

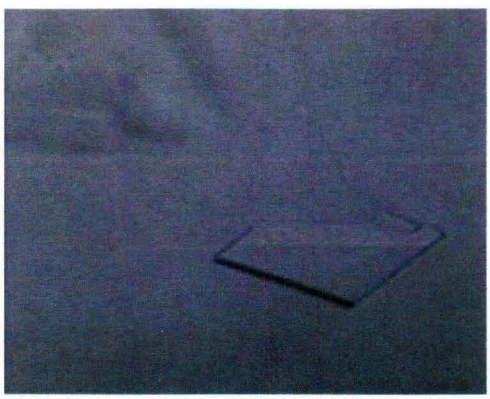


(b)

Fig. 2.3.2 (a) , (b) EhCaBP2 crystallization condition 45–60 % (w/v) MPD, 5 mM $CaCl_2$, 50 mM acetate.

(c)

Fig. 2.3.2 (c) Sr–EhCaBP2 crystallization condition 40–50 % (w/v) MPD, 50 mM acetate buffer pH 4.6, 5 mM $SrCl_2$.



(d)

Fig. 2.3.2 (d) IQ1–EhCaBP2 crystallization condition 30 % (w/v) ethanol, 40–50 % (w/v) MPD, 50 mM cacodylate buffer pH 6.5.

Table 2.3.4 (values in parentheses are for the last resolution shell)

Data Collection Statistics		
	EhCaBP2	Sr-EhCaBP2
X ray source	BNL X9	Chess A1
Wavelength (A°)	1.25473	0.9764
Space Group	$P2_1$	$P2_1$
Unit - cell parameters		
a (A°)	111.74	69.2
b (A°)	68.8	112
c (A°)	113.25	93.4
β(°)	116.7	92.8
Resolution range	30.0-2.5	20.0-2.68
R_{sym} (%)	5.2	8.5(36.0)
Completeness (%)	87.9	88.1(66.8)
Total number of observations	565291	447423
No of unique observations	47410	37740
Redundancy	11.9	5.9
Average I/σ(I)	22.5	10.2(3.3)
Crystal mosaicity (°)	0.4	0.44

Table 2.3.5 (values in parentheses are for the last resolution shell)

Data Collection Statistics	
	IQ1-EhCaBP2
X ray source	SSRL 11-1
Wavelength (A°)	0.9764
Space Group	$P2_1$
Unit - cell parameters	
a (A°)	60.5
b (A°)	69.86
c (A°)	86.5
β(°)	97.9
Resolution range	50.0-3.11
R_{sym} (%)	4.5(32.5)
Completeness (%)	94.8(95.7)
Total number of observations	497664
No of unique observations	13758
Redundancy	36.2
Average I/σ(I)	19.9(3.8)
Crystal mosaicity (°)	1.5

EhCaBP2 purity was assayed on SDS – PAGE (fig. 2.3.1) and found it to be 99 % pure. Only these fractions containing 99 % pure EhCaBP2 were taken for crystallization trials. The initial trials of EhCaBP2 crystallization gave high- mosaicity poorly diffracting crystals in 45–65 % methylenepentanediol (MPD) with 5 mM $CaCl_2$ and 50 mM acetate buffer pH 4.6 using the hanging drop vapor - diffusion method at 289 K. In a subsequent crystallization experiment, EhCaBP2 was crystallized under similar conditions in the presence of 1 mM strontium (Table 2.3.3) as an additive [(fig. 2.3.2 (b)]. These crystals diffracted to 2.6 A° resolution and belonged to space group P21, with unit-cell parameters a = 111.74, b = 68.83 c = 113.25 A°, β = 116.7°.

Prior to crystallization, the Sr–EhCaBP2 complex was concentrated to 15 mg/ ml and crystallized with 40–50 % MPD as precipitant in 50 mM acetate buffer pH 4.6 and 5 mM $SrCl_2$ using the hanging drop vapour - diffusion method at 289 K. At higher concentrations of MPD, fast-growing plate-like poly-microcrystals of the Sr–EhCaBP2 complex were formed. These crystals were used for microseeding. The best diffracting crystals appeared in 10–15 d at 42 % MPD when the protein and precipitant were mixed in a 2.5:1 ratio; after microseeding, the concentration of precipitant was increased to 60 % [fig. 2.3.2 (c)]. The crystals grew to approximately 0.5 x 0.5 x 0.1 mm in about 7–10 d. The crystals were frozen in 65 % MPD, 10 mM cacodylate, 25 mM acetate pH 4.6, 5 mM $SrCl_2$. The crystals diffracted to 2.55 A° and belonged to space group P21, with unit-cell parameters a = 69.18, b = 112.03, c = 93.42 A°, β = 92.8°. The data-collection statistics are summarized in (Table 2.3.4). Based on a Matthews coefficient calculation (Matthews, 1968), each asymmetric unit in this cell could contain 7–11 molecules (V_M is in the range 3.4–2.2 $A^{\circ 3}$ Da^{-1}), with a solvent content ranging from 64 to 43.4 %, respectively. The C-terminal domain of calmodulin from paramecium (PDB code 1exr; Wilson & Brunger, 2000) was used as model for molecular replacement and seven peaks were obtained using MOLREP (Vagin &Teplyakov, 1997).

This IQ1–EhCaBP2 complex was concentrated to 15 mg/ml and crystallized using 50 mM cacodylate buffer pH 6.4 with 30 % MPD, 30–45 % ethanol as precipitants. All these crystals were obtained at a temperature of 289 K [fig. 2.3.2 (d)]. These crystals were of

poor quality with layers and cracks; very few crystals were good-looking. The better looking crystals were transferred into cryoprotectant solutions containing mother liquor with an MPD concentration increased by 5 % from the crystallization condition prior to data collection. These crystals diffracted to 3.0 A° and belonged to space group P21 with unit-cell parameters a = 60.5, b = 69.86, c = 86.5 A°, β = 97.9°. The data collection statistics are summarized in (Table 2.3.5). Based on a Matthews coefficient calculation (Matthews, 1968), each asymmetric unit in this cell can contain three to five molecules (V_M in the range 3.8–2.3 $A^{\circ 3}$ Da^{-1}), with a solvent content ranging from 67 to 45 % respectively.