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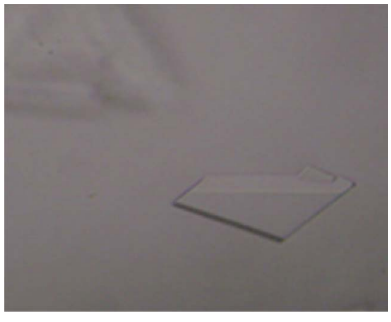
Crystallization and preliminary crystallographic analysis of calcium-binding protein-2 from *Entamoeba histolytica* and its complexes with strontium and the IQ1 motif of myosin V

Calcium plays a pivotal role in the pathogenesis of amoebiasis, a major disease caused by *Entamoeba histolytica*. Two domains with four canonical EF-hand-containing calcium-binding proteins (CaBPs) have been identified from *E. histolytica*. Even though they have very high sequence similarity, these bind to different target proteins in a Ca^{2+}-dependent manner, leading to different functional pathways. Calcium-binding protein-2 (EhCaBP2) crystals were grown using MPD as a precipitant. The crystals belong to space group $P2_1$, with unit-cell parameters a = 111.74, b = 68.83, c = 113.25 Å, β = 116.7°. EhCaBP2 also crystallized in complex with strontium (replacing calcium) at similar conditions. The crystals belong to space group $P2_1$, with unit-cell parameters a = 69.18, b = 112.03, c = 93.42 Å, β = 92.8°. Preliminary data for EhCaBP2 crystals in complex with an IQ motif are also reported. This complex was crystallized with MPD and ethanol as precipitating agents. These crystals belong to space group $P2_1$, with unit-cell parameters a = 60.5, b = 69.86, c = 86.5 Å, β = 97.9°.

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) monomeric 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 (Gly63 and Gly67) 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.



They bind different sets of *E. histolytica* target proteins in a Ca^{2+}-dependent manner (Chakrabarty *et al.*, 2004).

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 signalling 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)Q*xxx*R*xxxx*(R,K) (reviewed by Bahler & Rhoads, 2002). In order to understand the binding of EhCaBP2 to IQ motifs and the nature of the interactions, we have taken the IQ1 motif of myosin V from *Saccharomyces cerevisiae* (Myo2P) and initiated the study of the three-dimensional structure of the complex between IQ1 and EhCaBP2 (IQ1–EhCaBP2).

To understand the flexibility of the calcium-binding loops in EhCaBP2, we have replaced calcium with strontium and initiated the three-dimensional structure determination of the strontium–EhCaBP2 complex (Sr–EhCaBP2). In this study, we present the preliminary X-ray crystallographic data on crystals of EhCaBP2 in complex with the IQ1 motif and in complex with Sr^{2+}.

2. Experimental procedures

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

The purification of EhCaBP2 was performed as described previously (Prasad *et al.*, 1993). This protein was further purified using phenyl Sepharose affinity chromatography. The protein was dialyzed in 5 m*M* $CaCl_2$, 50 m*M* 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 m*M* NaCl with 50 m*M* HEPES buffer pH 7.5, 5 m*M* $CaCl_2$ to remove non-specific bound proteins and the column was washed with 5 m*M* $CaCl_2$, 50 m*M* HEPES buffer pH 7.5. The EhCaBP2 was eluted with 5 m*M* EGTA, 10 m*M* EDTA in 25 m*M* cacodylate buffer pH 5.0.

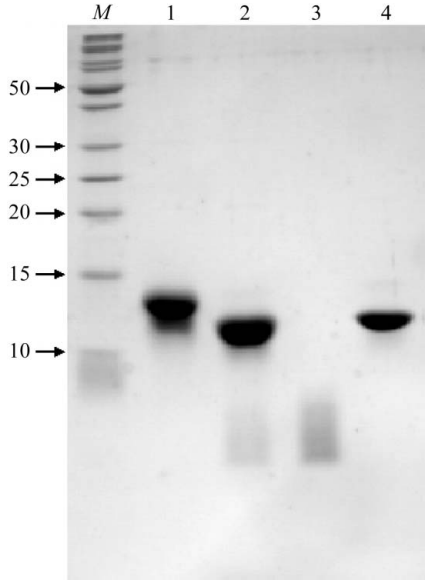


Figure 1
SDS–PAGE. The following samples were subjected to 14% SDS–PAGE and visualized using Coomassie blue staining. Lane *M*, standard molecular-weight markers (kDa). Lane 1, supernatant of EhCaBP2 and IQ1 without calcium in 1 m*M* EGTA. Lane 2, supernatant of EhCaBP2 and IQ1 in the presence of calcium. Lane 3, IQ1 peptide. Lane 4, pure EhCaBP2.

Table 1
Crystallization of EhCaBP2 and its complexes.

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

2.2. Preparation of Sr–EhCaBP2 complex

The purified EhCaBP2 was dialyzed against 6 *M* urea, 10 m*M* EDTA to remove the bound Ca^{2+} from the EhCaBP2. Subsequently, this apo-form EhCaBP2 was dialyzed against 10 m*M* cacodylate buffer with 5 m*M* $SrCl_2$ with two buffer changes to form the Sr–EhCaBP2 complex.

2.3. 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 m*M* acetate buffer pH 3.5. This mixture of IQ1 and EhCaBP2 was dialyzed against 50 m*M* cacodylate pH 5.0, 5 m*M* $CaCl_2$. However, when the pH was raised to 5.0 some precipitate was observed in the dialysis membrane. When a similar experiment was performed without calcium, more precipitate was observed in the dialysis membrane. These two samples were centrifuged and the supernatants were analyzed by SDS–PAGE (Fig. 1). IQ1–EhCaBP2 in the presence of calcium showed two bands corresponding to EhCaBP2 and IQ1, while without calcium it showed one band corresponding to EhCaBP2. It became clear that 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 using Centricon microconcentration devices (Amicon Inc. Beverly, MA, USA) and used for crystallization.

2.4. 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 vapour-diffusion method. The crystallization trials were performed using different ratios of protein and precipitant solution (1:1, 1.5:1, 2:1, 3:1) and then equilibrated by vapour diffusion with same precipitant. The details of crystallization conditions for EhCaBP2 and its complexes are summarized in Table 1.

2.5. 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 beamline X9. The data from Sr–EhCaBP2 crystals were collected at CHESS using the A1 beamline. The data from IQ1–EhCaBP2 crystals were collected at SSRL beamline 11-1. All the data sets were indexed and scaled with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

The initial trials of EhCaBP2 crystallization gave high-mosaicity poorly diffracting crystals in 45–65% methylenepentanediol (MPD) with 5 m*M* $CaCl_2$ and 50 m*M* acetate buffer pH 4.6 using the hanging-drop vapour-diffusion method at 289 K. In a subsequent crystallization experiment, EhCaBP2 was crystallized under similar conditions in the presence of 1 m*M* strontium (Table 1) as an additive (Fig. 2*b*). These crystals diffracted to 2.6 Å resolution and belonged to space group $P2_1$, with unit-cell parameters a = 111.74, b = 68.83, c = 113.25 Å, β = 116.7°.

Prior to crystallization, the Sr–EhCaBP2 complex was concentrated to 8 mg ml^{-1} and crystallized with 40–50% MPD as precipitant in 50 m*M* acetate buffer pH 4.6 and 5 m*M* $SrCl_2$ 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*c*). The crystals grew to approximately 0.5 × 0.5 × 0.1 mm in about 7–10 d. The crystals were frozen in 65% MPD, 10 m*M* cacodylate, 25 m*M* acetate pH 4.6, 5 m*M* $SrCl_2$. The crystals diffracted to 2.55 Å and belonged to space group $P2_1$, with unit-cell parameters a = 69.18, b = 112.03, c = 93.42 Å, β = 92.8°. The data-collection statistics are summarized in Table 2. 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 Å 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 & Brünger, 2000) was used as model for molecular replacement and seven peaks were obtained using *MOLREP* (Vagin & Teplyakov, 1997). Model building and refinement are in progress.

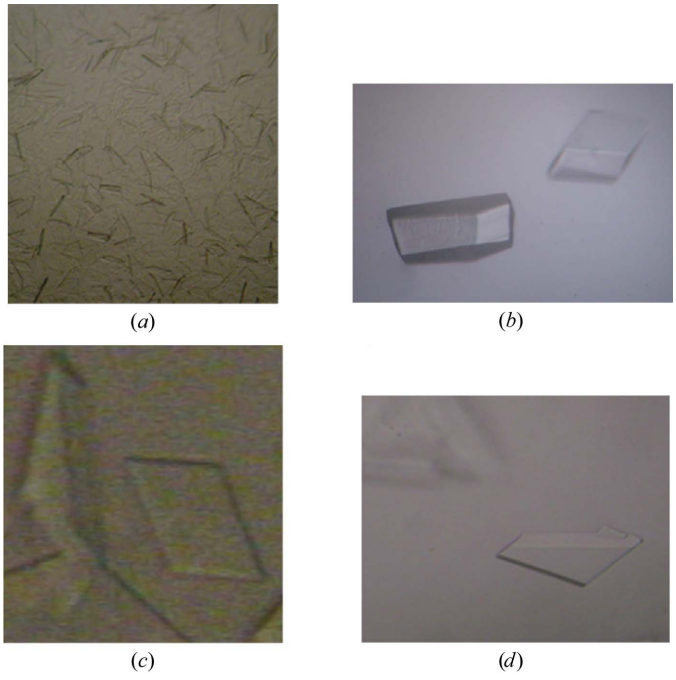


Figure 2
(*a*) Microcrystals of EhCaBP2. (*b*) Improved crystals of EhCaBP2 after microseeding. (*c*) Crystals of Sr–EhCaBP2. (*d*) Crystals of IQ1–EhCaBP2. Each IQ-motif peptide binds to one EhCaBP2. The sequence of the IQ1 motif from Myo2P is ^{783}KMHNSIVMIQKKIRAKYYRKQYL^{805}. The best-looking crystals of EhCaBP2 grew to dimensions of 0.6 × 0.3 × 0.2 mm and those of Sr–EhCaBP2 typically grew to 0.4 × 0.4 × 0.1 mm. The approximate dimensions of the IQ1–EhCaBP2 crystals are 0.2 × 0.15 × 0.05 mm.

Table 2
Data-collection statistics.

Values in parentheses are for the last resolution shell.

	EhCaBP2	Sr–EhCaBP2	IQ1–EhCaBP2
X-ray source	BNL X9	Chess A1	SSRL 11-1
Wavelength (Å)	1.25473	0.9764	0.9764
Space group	$P2_1$	$P2_1$	$P2_1$
Unit-cell parameters			
a (Å)	111.74	69.2	60.5
b (Å)	68.8	112.0	69.86
c (Å)	113.25	93.4	86.5
β (°)	116.7	92.8	97.9
Resolution range	30.0–2.5	20.0–2.68	50.0–3.11
R_{sym} (%)	5.2	8.5 (36.0)	4.5 (32.5)
Completeness (%)	87.9	88.1 (66.8)	94.8 (95.7)
Total No. of observations	565291	447423	497664
No. of unique observations	47410	37740	13758
Redundancy	11.9	5.9	36.2
Average $I/\sigma(I)$	22.5	10.2 (3.3)	19.9 (3.8)
Crystal mosaicity (°)	0.4	0.44	1.5

This IQ1–EhCaBP2 complex was concentrated to ~8 mg ml^{-1} and crystallized using 50 m*M* 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*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 Å and belonged to space group $P2_1$ with unit-cell parameters a = 60.5, b = 69.86, c = 86.5 Å, β = 97.9°. The data-collection statistics are summarized in Table 2. 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), with a solvent content ranging from 67 to 45%, respectively. The structure solution is in progress.

The structure of EhCaBP2 will provide detailed information on how it differs structurally from EhCaBP1 and as a consequence how it differs functionally. The complex of EhCaBP2 with IQ1 of Myo2P will show how it binds to IQ motifs and thus begin to explain the differences in the specificity of CaBPs for the target regions.

We thank Peter Zwart for collecting the data on EhCaBP2 at BNL and Mark Wilson for data collection on IQ1–EhCaBP2 at SSRL. SG thanks Professor Carolyn Cohen for allowing the time to collect the data on Sr–EhCaBP2 at CHESS and to transport the crystals to BNL. We would like to thank the staff members of MacCHESS, BNL and SSRL for assistance during data collection. The authors thank the Department of Science and Technology, Government of India for funding.

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