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Expression, purification and crystallization of a functional core of the voltage-dependent calcium channel β subunit

Yarden Opatowsky, Orna Chomsky-Hecht and Joel A. Hirsch*

Department of Biochemistry, Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel

Correspondence e-mail: jhirsch@post.tau.ac.il

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Two versions of the functional core of the rabbit voltage-dependent calcium channel β2a subunit were expressed in *Escherichia coli*. These proteins were purified to homogeneity and screened for crystallization. Crystallization conditions were refined using the hanging-drop vapour-diffusion method and two crystal forms were pursued. Crystal form I is represented by thick rods with tetragonal symmetry, unit-cell parameters $a = b = 75$, $c = 165$ Å and a diffraction limit of 3.4 Å which were obtained using ammonium sulfate as a precipitant. Crystal form II gives rise to plates with orthorhombic symmetry, unit-cell parameters $a = 35$, $b = 75$, $c = 165$ Å and a diffraction limit of 2.3 Å which were grown using polyethylene glycol 20K as a precipitant.

1. Introduction

Voltage-dependant calcium channels (VDCCs) are key components in excitation–contraction and excitation–secretion coupling and other vital signalling systems. In response to electrical stimuli, the VDCC opens and allows a flow of calcium ions to enter the cell. In addition to an α1 pore-forming unit, the VDCC is composed of additional auxiliary subunits, namely a cytoplasmic β subunit and membrane-spanning γ and $\alpha 2\delta$ subunits. These components modulate the channel's membrane expression, opening probability, inactivation kinetics and current-to-voltage ratios. Four genes encode the β subunit, designated β1–4, and each expresses several splice-variants (Arikkath & Campbell, 2003). Functional features of the β subunit include increased peak amplitude, a hyperpolarizing shift in activation and changes in the inactivation kinetics (Walker & De Waard, 1998).

The β subunits of different organisms and types vary in size (52–88 kDa), but share a common architecture of five sequence regions. Two structural domains (I and II) have been defined biochemically by limited proteolysis, mass-spectrometric analysis and N-terminal sequencing (Opatowsky *et al.*, 2003). The domain borders coincide with the two regions of conservation in the VDCC β gene family. A variable linker separates these two domains; however, it is clear from our previous work (Opatowsky *et al.*, 2003) that the two domains are bound to each other in a stable fashion with or without the linker. Moreover, a two-domain complex, dubbed the linkerless core, is electrophysiologically active.

The β subunit associates with the α1 pore-forming unit mainly through a high-affinity interaction site (Pragnell *et al.*, 1994). This site, named the AID (α1-interacting domain), is located in the α1 cytoplasmic loop connecting the first and second membrane domains and consists of 18 amino acids, nine of which are totally conserved. The BID (β-interacting domain) is a 30-amino-acid sequence at the beginning of the second conserved domain (domain II) of the β subunit.

In order to better understand the vital role of the VDCC β subunit in calcium signalling, we pursued the crystallization of the functional core of the β2a protein (Swiss-Prot accession No. P54288-2). Combined with electrophysiological and biochemical data, the crystal structure determination of this engineered protein should help us to achieve this goal. Here, we present the crystallization conditions and preliminary X-ray analysis of the β functional core, the initial steps in the structure determination of the β subunit.

2. Experimental conditions

2.1. Subcloning, expression and purification

The VDCC β2a linkerless core construct was subcloned into a modified pET21-d vector and expressed as previously described (Opatowsky *et al.*, 2003). A VDCC β2a truncated linker core construct, *i.e.* domain I fused to domain II, was prepared as follows. Subcloning was sequential. PCR was used to engineer *Bam*HI and *Eco*RI restriction sites into the β2a domain I-encoding gene. The primers used were the following: sense, 5′-GCGCGGATCCAG-CCGTCCATCCGATTCAGATGTG-3′; antisense, 5′-CGCGGAATTCCTTTGCTCTCT-GTTCATGCTGTAG-3′. The PCR product was ligated into a doubly digested (*Bam*HI,

*Eco*RI) pET21-d vector. Next, *Not*I and *Eco*RI sites were introduced into the domain II fragment by PCR using the sense primer 5′-CGGAATTCAAGCTTCACTC-CAAAGAGAAAAGAATGCCC-3′ and the antisense primer 5′-TTATACTAGC-GGCCGCTCAAAGGAGAGGGTTGGG-GAGATT-3′. Finally, the domain II PCR product was ligated into a doubly digested (*Eco*RI, *Not*I) pET21-d vector which already contained the domain I-encoding fragment. Positive clones were identified by restriction analysis and sequencing. The resulting gene encodes domain I fused to domain II with four residues (EFKL) between them that arise owing to the cloning procedure.

Expression of the proteins was essentially as described previously (Opatowsky *et al.*, 2003). In brief, protein was expressed in transformed Tuner (Novagen) *Escherichia coli* bearing the CodonPlus (Stratagene) plasmid, grown in 2× YT media containing 100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol at 289 K for 14 h. Cells were then harvested by centrifugation and frozen for subsequent use. Cell paste was suspended in 100 ml lysis buffer (300 m*M* NaCl, 50 m*M* sodium phosphate pH 8, 1 mg DNase). After lysis using a French press, cell debris was removed by centrifugation at 20 000*g*. The soluble fraction was loaded onto a pre-equilibrated metal-chelate Ni-CAM (Sigma) column (buffer *A*: 300 m*M* NaCl, 50 m*M* sodium phosphate pH 8) and washed with buffer *A* supplemented with 7 m*M* imidazole until a stable baseline was achieved. The protein was eluted with buffer *A* supplemented with 150 m*M* imidazole, diluted sixfold with 10% glycerol and loaded onto a Q-Sepharose (Amersham Pharmacia) column pre-equilibrated with buffer *B* (70 m*M* NaCl, 20 m*M* sodium phosphate pH 8). Fractions were eluted with a shallow gradient of buffer *C* (400 m*M* NaCl, 20 m*M* sodium phosphate pH 8). VDCC β2a-containing fractions (180–220 m*M* NaCl) were pooled and subjected to TEV protease digestion in order to remove the 8×His tag. Proteolysis continued for 12 h and the protein was subsequently loaded onto a hydroxylapatite (Calbiochem) column pre-equilibrated with buffer *D* (200 m*M* NaCl, 50 m*M* sodium phosphate pH 8) and washed with buffer *D* until a flat baseline was achieved. The protein was then eluted with buffer *E* (200 m*M* NaCl, 200 m*M* potassium phosphate pH 8, 5 m*M* β-mercaptoethanol) and applied to a pre-equilibrated Superdex-200 gel-filtration column (Amersham Pharmacia) with buffer *F* (200 m*M* NaCl, 20 m*M* Tris pH 8, 5 m*M* β-mercaptoethanol). The elution peak was concentrated to 12 mg ml^{-1} using spin concentrators (Vivascience), divided into aliquots and flash-frozen in liquid N_2 (Fig. 1).

2.2. Crystallization

Initial screens of both proteins were performed at 277 and 292 K with Hampton Crystal Screen and Crystal Screen 2 (Hampton Research) in 96-well sitting-drop plates (Corning). The drop size was 2 µl, with a sample:reservoir ratio of 1:1. After 24 h, microcrystals and small crystals appeared using many high-molecular-weight PEG and ammonium sulfate conditions. Crystallization was refined using hanging-drop vapour-diffusion plates by varying different precipitant concentrations *versus* different pHs and buffers. The addition of low concentrations of alcohols and salts, both at 277 and 292 K, gave optimal growth conditions for both the truncated linker core and linkerless core forms. Both protein types gave thick rod-shaped crystals using 1.6 *M* ammonium sulfate, 0.1 *M* HEPES pH 7, 5 m*M* β-mercaptoethanol at 277 K (crystal form I). Crystals appeared after several hours and diffracted for 48 h post-setup. The truncated linker core protein also gave plate-shaped crystals using 3% PEG 20000 (Fluka), 0.1 *M* Bicine pH 9, 100 m*M* NaCl, 5 m*M* β-mercaptoethanol at 292 K (crystal form II). These crystals appeared after 12 h and diffracted for no longer than 36 h post-setup (Fig. 2).

2.3. Data collection

Prior to flash-freezing in cryoloops, crystals were gradually transferred to cryoprotecting solutions, containing, in addition to cryoprotectant (30% sucrose for the ammonium sulfate crystals and 35% glycerol for the PEG crystals), all of the mother-liquor components. Heavy-atom-soak crystals were prepared by adding a final concentration of 1 m*M* heavy-atom solution to the mother liquor for 5 min prior to the cryoprotectant soak. Flash-frozen crystals were then placed on the 110 K N_2-cooled goniometer head for data collection. Using a

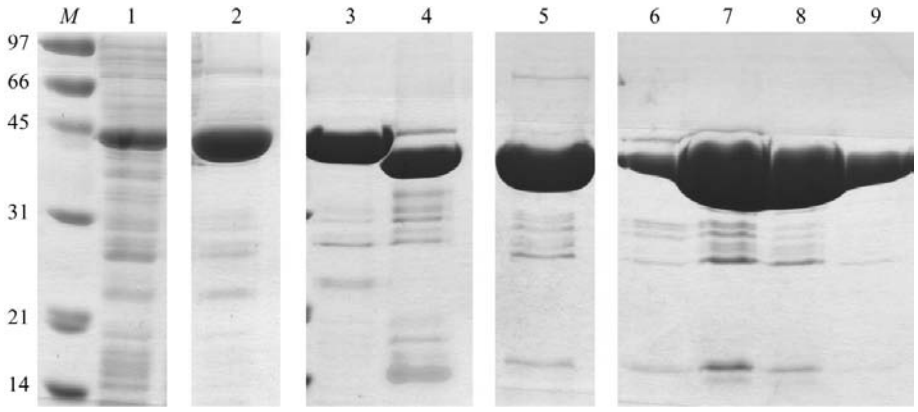

Figure 1
SDS–PAGE of the truncated linker core construct purification steps. Lane *M*, molecular-weight markers (kDa); lane 1, crude extract; lane 2, Ni-CAM elulate; lane 3, Q-Sepharose eluate; lane 4, after TEV digestion; lane 5, hydroxylapatite eluate; lanes 6–9, gel-filtration eluate. The gel-filtration eluate was used for crystallization.

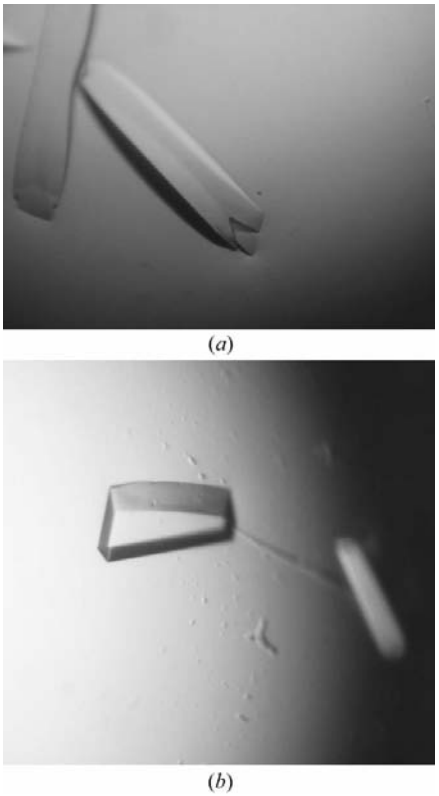

Figure 2
(*a*) Rod-shaped form I and (*b*) plate-like form II crystals of voltage-gated calcium channel β subunit (truncated linker core construct). Form I crystals are approximately 1.25 mm in length, while form II crystals are about 0.5 mm in the longest dimension.

Table 1
Data-processing statistics for the voltage-gated calcium channel β-subunit crystals.

Values in parentheses are for the highest resolution shells. Data were collected at the ESRF, Grenoble, France.

Protein	Truncated linker core	Truncated linker core	Linkerless core
Crystal form	Form II	Form I	Form I
Wavelength (Å)	0.933	0.976	0.976
Space group	$P2_12_12$	$P4_12_12$	$P4_12_12$
Unit-cell parameters (Å)			
a (Å)	74.1	75.6	76.5
b (Å)	163.8	75.6	76.5
c (Å)	34.8	164.4	165.1
$\alpha = \beta = \gamma$ (°)	90	90	90
Resolution range (Å)	50–2.3 (2.38–2.3)	50–3.55 (3.68–3.55)	50–3.55 (3.68–3.55)
Total reflections	71732	26270	45028
Unique reflections	18271	6029	6447
Completeness (%)	92.8 (87.9)	96.2 (97.5)	100 (100)
$R_{\rm merge}$ (%)	4.8 (31)	7.4 (49.7)	10.7 (47.6)
$I/\sigma(I)$	15.4 (4.3)	17.8 (3.9)	18.7 (5.2)
Beamline	ID14-2	BM-14	BM-14

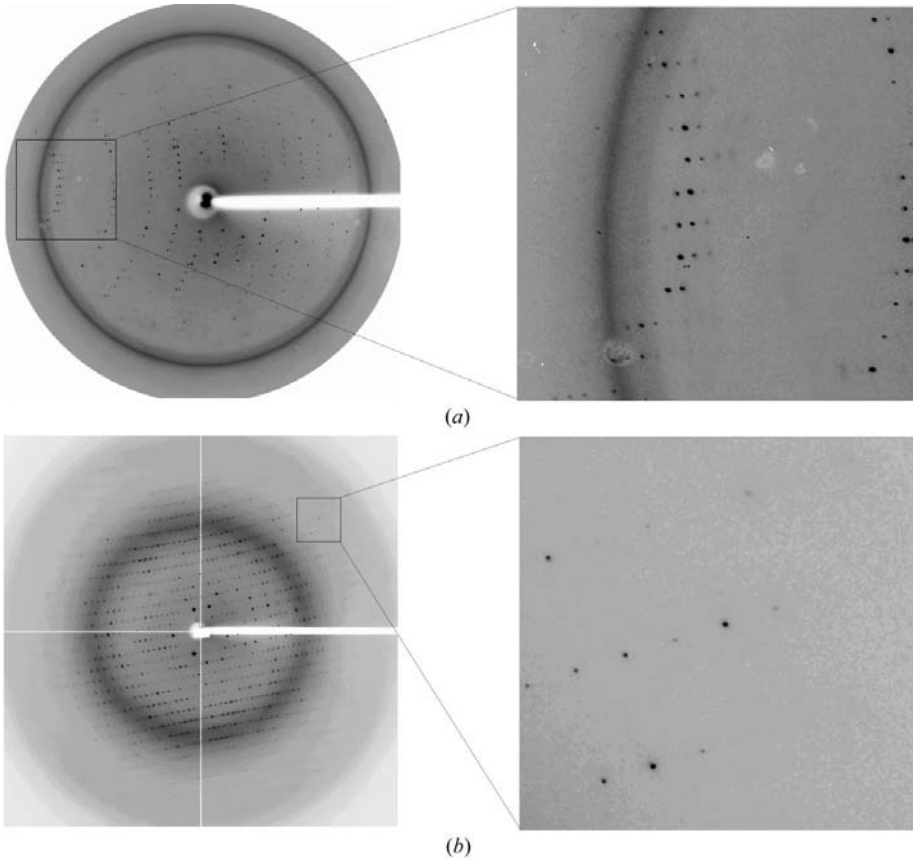

Figure 3
Oscillation frames (0.5°) of (*a*) form I and (*b*) form II crystals of the truncated linker core protein. Diffraction data were observed to 3.4 and 2.3 Å, respectively.

rotating Cu-anode X-ray source with a Rigaku R-AXIS IV image-plate detector system, crystals were screened for diffraction quality. Synchrotron data collection was conducted at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. At beamline ID14-2, data were collected using an ADSC Q4 CCD detector, while at beamline BM-14, a MAR CCD detector was utilized. All diffraction data were processed with *HKL* (Otwinowski & Minor, 1997) and the statistics are shown in Table 1.

3. Results and discussion

Functional cores of the voltage-dependent calcium channel β subunit (β2a isoform) were expressed in *E. coli*, purified to homogeneity and crystallized using two different constructs. Two crystal forms predominated. While crystal form II ($d_{\rm min}$ = 2.3 Å) should provide us with a detailed atomic structure, form I ($d_{\rm min}$ = 3.55 Å) may provide structural information about the conformational variability of these two-domain molecules (Fig. 3).

To date, attempts to obtain experimental phasing by isomorphous replacement have been thwarted as crystal form II suffers from significant non-isomorphism, as evident by large deviations in the unit-cell size along the longest axis and by the high χ^2 values (>20) obtained by scaling independent data sets, including native *versus* native sets. In order to obtain experimental phase information, MAD experiments will be performed on recombinant selenomethionine protein crystals. Substituted protein has already been prepared and crystallized.

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References

Arikkath, J. & Campbell, K. P. (2003). *Curr. Opin. Neurobiol.* **13**, 298–307.
Opatowsky, Y., Chomsky-Hecht, O., Kang, M. G., Campbell, K. P. & Hirsch, J. A. (2003). *J. Biol. Chem.* **27**, 52323–52332.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Pragnell, M., De Waard, M., Mori, Y., Tanabe, T., Snutch, T. P. & Campbell, K. P. (1994). *Nature (London)*, **368**, 67–70.
Walker, D. & De Waard, M. (1998). *Trends Neurosci.* **21**, 148–154.