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Crystallization and preliminary X-ray diffraction analysis of calexcitin from *Loligo pealei*: a neuronal protein implicated in learning and memory

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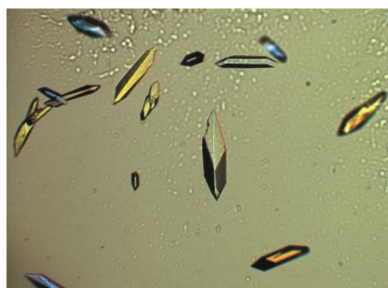
The neuronal protein calexcitin from the long-finned squid *Loligo pealei* has been expressed in *Escherichia coli* and purified to homogeneity. Calexcitin is a 22 kDa calcium-binding protein that becomes up-regulated in invertebrates following Pavlovian conditioning and is likely to be involved in signal transduction events associated with learning and memory. Recombinant squid calexcitin has been crystallized using the hanging-drop vapour-diffusion technique in the orthorhombic space group $P2_12_12_1$. The unit-cell parameters of $a = 46.6$, $b = 69.2$, $c = 134.8$ Å suggest that the crystals contain two monomers per asymmetric unit and have a solvent content of 49%. This crystal form diffracts X-rays to at least 1.8 Å resolution and yields data of high quality using synchrotron radiation.

1. Introduction

The protein calexcitin was originally identified in the photoreceptor neurons of the marine snail *Hermissenda crassicornis* as a ~20 kDa protein that became up-regulated and phosphorylated following a Pavlovian training protocol (Nelson *et al.*, 1990). *Hermissenda* are normally attracted towards light but are repelled by movement, which causes them to retract. Interestingly, they become conditioned to move away from light (normally an attractive stimulus) if they are simultaneously exposed to both light and movement (*e.g.* on an illuminated turntable). The up-regulation of calexcitin is correlated with exposure to both light and movement and not with each separate stimulus alone. Detailed electrophysiological studies showed that micro-injection of purified calexcitin protein into *Hermissenda* photoreceptors reduced early and late voltage-dependent K^+ currents to a similar extent as that caused by the Pavlovian conditioning experiment itself (Alkon & Nelson, 1990). Thus, calexcitin was shown to regulate the voltage-dependent potassium channel and the calcium-dependent potassium channel and was capable of reproducing the electrophysiological effects of learning when injected into *Hermissenda* neurons. The protein was found to bind calcium and there is evidence that it has GTPase activity (Nelson *et al.*, 1990). Recent reports suggest that the GTPase activity of calexcitin is stimulated by calcium in several invertebrate species, most notably *Drosophila* (Nelson *et al.*, 2003).

Calexcitin has also been isolated from the optic lobe of the long-finned squid *Loligo pealei* (Nelson *et al.*, 1994, 1996). Cloning and sequencing of squid calexcitin cDNA showed that the protein consists of 191 amino acids and has sequence similarity with several sarcoplasmic calcium-binding proteins (SCPs) and some elements of sequence similarity with GTP-binding proteins of the ADP-ribosylation factor family (Nelson *et al.*, 1996; Gombos *et al.*, 2001). Squid calexcitin has significant sequence identity (28%) with an invertebrate SCP of known X-ray structure, referred to as ASCP (an SCP from the proto-vertebrate marine organism *Amphioxus*; Cook *et al.*, 1993). This similarity suggests that calexcitin contains up to four EF-hand motifs. Calcium-binding assays indicated that two or three calcium ions were bound per monomer of squid calexcitin and circular dichroism (CD) as well as other spectroscopic studies showed that calcium binding causes conformational changes in the molecule (Ascoli *et al.*, 1997; Gombos *et al.*, 2001).



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Monoclonal antibodies to squid calexcitin cross-react with a protein of the same molecular weight in mammalian neuronal cells (Nelson *et al.*, 1994). Electrophysiological studies showed that low concentrations of squid calexcitin had a strong inhibitory effect on human K^+ channels (Nelson *et al.*, 1996). These findings suggest that a functionally related protein exists in mammals and may play a similar role in the regulation of potassium channels. It has been shown that calexcitin is phosphorylated by protein kinase C, which causes it to translocate to the cell membrane where its effects on membrane excitability can be exerted (Nelson & Alkon, 1995). In addition, it has been shown that calexcitin causes the release of calcium from the endoplasmic reticulum (ER) by binding to and activating ryanodine receptors (Cavallaro *et al.*, 1997; Nelson *et al.*, 1999). The latter constitute one of the main channels for Ca^{2+} release from the ER and have also been implicated in associative learning.

2. Materials and methods

2.1. Cloning, overexpression and purification of squid calexcitin

We obtained a pET16b *Escherichia coli* expression construct of squid calexcitin cDNA (a kind gift from Professor Dan Alkon, then at NIH, Bethesda, USA) and we excised and re-ligated the gene into the pET11a vector, which lacks the N-terminal His_{10} tag of pET16b, in case the His tag presented problems either in its removal (which requires factor Xa cleavage) or in the crystallization process. Expression of both constructs (His-tagged and non-His-tagged protein) was attempted in *E. coli* BL21 (DE3) cells initially using 10 ml trial cultures and these yielded strong induction bands on SDS–PAGE gels of the expected molecular weights following induction of the cells with 1 m*M* isopropyl β-D-thiogalactopyranoside (IPTG).

The expression of both constructs was scaled up to 5 l and the resulting cells were lysed by sonication. This was followed by ultracentrifugation to remove the insoluble material and SDS–PAGE then established that virtually all of the calexcitin expressed by both constructs was soluble. Purification of the His-tagged protein from the supernatant involved use of a nickel-affinity column (Ni–NTA column, Qiagen) and this yielded pure calexcitin (as detected by SDS–PAGE) in a single chromatography step. Using this approach, around 70 mg of His-tagged calexcitin was obtained per litre of cell culture following induction with 1 m*M* IPTG for 6 h. Intriguingly, we found that storage of the His-tagged protein at 277 K for around one week resulted in a decrease in its molecular weight by ~4 kDa, presumably owing to the action of trace proteinases. Electrospray mass-spectrometric studies confirmed that this spontaneous degradation involved the removal of the His tag and amino acids forming the factor Xa recognition site, giving protein with a molecular weight (22 140 Da) that coincided to within 1 Da with that predicted from the amino-acid sequence of calexcitin lacking the N-terminal methionine. The second highest peak in the mass spectrum corresponded exactly with the molecular weight of the full-length squid calexcitin.

Protein of the same molecular weight was obtained from the pET11a construct which lacks the His tag, although in this case purification from the cell lysate involved fractional ammonium sulfate precipitation followed by gel-filtration HPLC *via* a Superdex 75 column (Amersham Biosciences). The protein obtained from this construct was only marginally less pure than the His-tagged protein from the nickel column as judged by SDS–PAGE and was obtained in similar if not greater yield (~90 mg per litre of culture following induction with 1 m*M* IPTG for 12 h).

Purified protein obtained from both constructs was concentrated in an Amicon stirred pressure cell and stored in 20 m*M* Tris–HCl pH 8.0, 1 m*M* dithiothreitol (DTT) and 1 m*M* $CaCl_2$. To prevent further degradation of the protein, a proteinase-inhibitor cocktail (Sigma) was added to the samples. Electrospray mass spectrometry showed that this resulted in an increase in the molecular weight of a large proportion of the protein by 183 Da. We interpreted this as a covalent modification by 4-(2-aminoethyl)benzenesulfonyl fluoride, a serine proteinase inhibitor that is present in the cocktail. Modification by this reagent would be expected to give rise to the observed molecular-weight increase. The finding that calexcitin appears to be modified by this compound suggests that the protein possesses an appropriately reactive serine residue.

2.2. Crystallization of squid calexcitin

The protein was used in commercial crystallization screens (Molecular Dimensions and Jena Bioscience) at a concentration of 3–4 mg ml^{-1}. For crystal screening, the hanging-drop method was

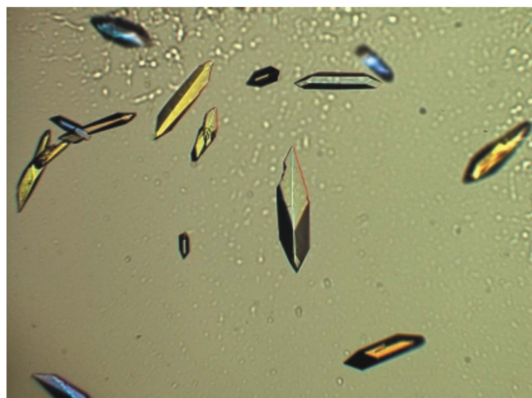

Figure 1
Crystals of recombinant squid (*L. pealei*) calexcitin which diffract to around 1.8 Å. The largest crystals shown here are approximately 0.5 mm in their largest dimension.

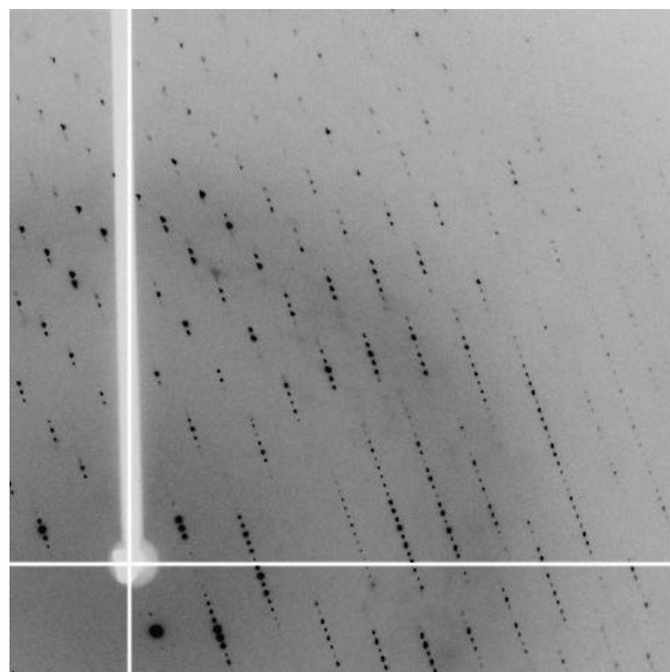

Figure 2
Part of one diffraction image obtained at ESRF (Grenoble) from a crystal of recombinant squid calexcitin.

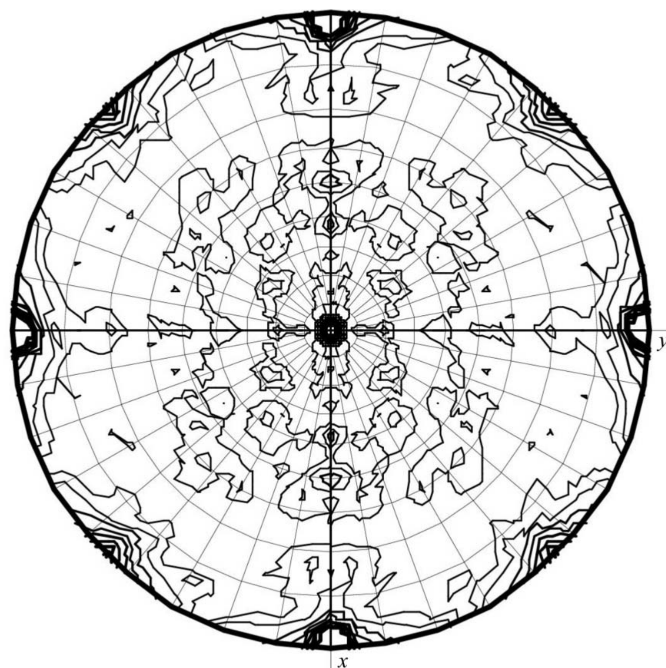

Figure 3
A χ = 180° section of the self-rotation function calculated from the squid calexcitin data set using a radius of integration of 20.0 Å and data to a resolution of 2.5 Å. The strongest peaks on the horizontal and vertical axes arise from space-group symmetry ($P2_12_12_1$). The other peaks on the circumference are around 1/3 of the height of the crystallographic twofold peaks and suggest the presence of non-crystallographic twofold axes perpendicular to *c* and oriented at approximately 45° from the *a* and *b* axes.

used with 5 µl of protein being mixed with 5 µl of each well solution on siliconized glass cover slips. Trial conditions were found and after optimization crystals of up to 1 mm in length (Fig. 1) were obtained in 30% polyethylene glycol (PEG) 4K, 0.2 *M* ammonium acetate and 0.1 *M* sodium citrate pH 5.0–6.5 at a protein concentration of 3 mg ml^{-1}. Crystals of recombinant squid calexcitin from either construct could be grown reproducibly using these conditions and were cryoprotected with 30% glycerol and mounted in mohair loops for freezing in liquid ethane and storage under liquid nitrogen.

2.3. X-ray diffraction analysis of squid calexcitin

The squid calexcitin crystals were taken to the European Synchrotron Radiation Facility (ESRF, Grenoble), where they were found to diffract to a resolution of around 1.8 Å (Fig. 2) using the ID14-1 beamline. Processing of this data set using *MOSFLM* (Leslie, 1992) and the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) indicates that the crystals belong to the orthorhombic space group $P2_12_12_1$ and have unit-cell parameters a = 46.6, b = 69.2, c = 134.8 Å. The processed data set extends to 1.8 Å resolution, with an overall $R_{\rm merge}$ of 6.1%, a completeness of 99.1% and a multiplicity of 9.0. Data-collection and processing statistics are given in Table 1. Use of the *MC* program (Collaborative Computational Project, Number 4, 1994) suggests that the crystals are likely to contain two calexcitin monomers (each of 22 kDa) per asymmetric unit, corresponding to a solvent content of 49%. A self-rotation function, calculated using *MOLREP* (Vagin & Teplyakov, 1997), indicates the presence of non-crystallographic twofold symmetry in the asymmetric unit (Fig. 3). Since efforts to determine the structure by molecular replacement using the structure of ASCP were not successful, preparation of selenomethionine-substituted protein for MAD analysis of the structure is in progress.

Table 1
Data-collection and processing statistics for recombinant *L. pealei* calexcitin.

Values for the outer resolution shell are shown in parentheses.

Beamline	ID14-1 (ESRF)
Wavelength (Å)	0.934
Space group	$P2_12_12_1$
Unit-cell parameters	
a (Å)	46.6
b (Å)	69.2
c (Å)	134.8
Matthews coefficient (Å^3 Da^{-1})	2.45
Resolution (Å)	48.3–1.8 (1.9–1.8)
$R_{\rm merge}$ (%)	6.1 (36.9)
Completeness (%)	99.1 (97.5)
Average $I/\sigma(I)$	22.5 (2.3)
Multiplicity	9.0 (3.8)

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