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Expression, purification, crystallization and preliminary X-ray diffraction studies of human liver regucalcin

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Regucalcin is a novel calcium ion (Ca^{2+}) binding protein that does not contain an EF-hand motif as a Ca^{2+}-binding domain and has been demonstrated to play a multi-functional role in many cell types. Human liver regucalcin, consisting of 299 amino-acid residues, was overexpressed in *Escherichia coli*, purified and crystallized by the vapour-diffusion method in the presence of polyethylene glycol 4000 as a precipitant. A native crystal diffracted to 2.8 Å with synchrotron radiation and belongs to space group $P2_1$, with unit-cell parameters a = 64.87, b = 52.52, c = 86.38 Å, β = 99.86°. Two molecules most probably exist in the asymmetric unit, corresponding to V_M = 2.2 Å^3 Da^{-1}. Heavy-atom derivative data were collected and the Pb derivative showed one high-occupancy site per molecule.

1. Introduction

The calcium ion (Ca^{2+}) plays an important role as an intracellular messenger. Ca^{2+} signals are mediated through Ca^{2+}-binding proteins such as calmodulin and protein kinase C and control many cell functions (Cheung, 1980; Williamson *et al.*, 1981; Reinhart *et al.*, 1984; Kraus-Friedman & Feng, 1996). In 1978, Yamaguchi and Yamamoto found a novel Ca^{2+}-binding protein, named regucalcin, in rat liver cells (Yamaguchi & Yamamoto, 1978). Regucalcin has only been identified in certain mammals such as human, monkey, rabbit, cow, mouse and rat. It is expressed in kidney, brain, heart and preferentially liver cells (Shimokawa *et al.*, 1995). It has been demonstrated that regucalcin plays a multi-functional role in many different tissues. For example, regucalcin maintains the intracellular Ca^{2+} concentration in terms of activating Ca^{2+}-dependent ATPases in the plasma membrane, microsomes and mitochondria of liver and kidney cells (Yamaguchi, 2000). Furthermore, as regucalcin has inhibitory effects on Ca^{2+}/calmodulin-dependent kinase, protein kinase C and protein phosphatases, which play important roles in intracellular Ca^{2+} signal transduction, it may function as an important regulatory protein in many cell types (Yamaguchi, 2000).

It has been demonstrated that regucalcin mRNA expression is enhanced in regenerating rat liver cells (Yamaguchi & Kanayama, 1995) and decreased in rat and human hepatoma cells (Murata *et al.*, 1997; Nakajima *et al.*, 1999; Inagaki *et al.*, 2000; Suzuki *et al.*, 2003) compared with its levels in normal liver cells. Transcript heterogeneity of the regucalcin gene has been identified in human hepatoma cells (Misawa & Yamaguchi, 2000*a*) and furthermore it has been shown that expression of oncogenes such as c-*myc* and Ha-*ras* are suppressed and the tumour-suppression gene *p53* is enhanced in the hepatoma cells of transgenic rats overexpressing regucalcin (Tsurusaki & Yamaguchi, 2003). These findings suggest that regucalcin may play a suppressive role in cells having high proliferative activity, preventing cell transformation into tumour cells. Enhancement of the regucalcin gene in tumour cells may be useful for cancer treatment by involvement of gene-therapy technology.

Interestingly, regucalcin does not contain an EF-hand motif as a Ca^{2+}-binding domain, thus differing from calmodulin and other Ca^{2+}-binding proteins (Shimokawa *et al.*, 1993; Misawa & Yamaguchi, 2000*b*). Hence, structural analysis of the protein is required to reveal the novel architecture of Ca^{2+} recognition and would be helpful in understanding the mechanism of intracellular Ca^{2+} signal transduction. Here we describe the cloning, expression, crystallization and preliminary X-ray diffraction study of human liver regucalcin, consisting of 299 amino-acid residues with a theoretical molecular weight of 33 kDa.

2. Material and methods

2.1. Protein cloning, expression and purification

Human liver regucalcin gene (EMBL/DDBJ/GenBank accession No. AB032064) was cloned into pGEX4T-2 vector (Amersham Pharmacia) by the *Bam*HI and *Eco*RI enzymes with a fused protein, glutathione *S*-transferase and a thrombin-cleavage site. After confirmation of the nucleotide sequence, *Escherichia*

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coli DH5α strain (Toyobo) carrying the resulting expression plasmid was grown overnight in LB medium containing 50 mg ml^{-1} ampicillin. The pre-culture was added to 3.6 l of SB medium containing ampicillin and incubated for 6 h at 310 K. Expression was induced with 1 m*M* isopropyl-1-β-D-thiogalactopyranoside overnight at 298 K. The cells were harvested by centrifugation and stored at 193 K.

Frozen cells were thawed, suspended in PBS including 0.5% Triton X-100 and 1 m*M* $CaCl_2$ and disrupted by sonication. After centrifugation at 10 000*g* for 30 min, the supernatant was mixed with glutathione Sepharose 4B (Amersham Pharmacia) and incubated for 3 h at 277 K. The resin was washed three times with buffer *A* (25 m*M* Tris–HCl pH 7.5, 1 m*M* $CaCl_2$) and incubated overnight with 60 U ml^{-1} thrombin (Amersham Pharmacia) dissolved in buffer *A*. The supernatant was dialyzed against 3 l buffer *B* (10 m*M* MES–NaOH pH 6.0, 1 m*M* $CaCl_2$) and applied onto a Mono-S column (Amersham Pharmacia) equilibrated with buffer *C* (25 m*M* MES–NaOH pH 6.0, 1 m*M* $CaCl_2$). Elution was performed with a linear 0–0.5 *M* NaCl gradient in buffer *C*. Fractions containing regucalcin were concentrated by Ultrafree centrifugal devices (Millipore) and then diluted with buffer *D* (25 m*M* Tris–HCl pH 8.0, 5 m*M* $CaCl_2$). This procedure was repeated several times. Finally, the sample was concentrated to 10 mg ml^{-1} and stored at 277 K.

2.2. Crystallization and heavy-atom derivatives preparation

Initial screening was performed at room temperature (293 K) by the sitting-drop vapour-diffusion method using Crystal Screens I and II (Hampton Research). Drops were prepared on a Strips Plate (Hampton Research) and equilibrated against 0.1 ml of reservoir solutions. The drop volume was 0.8 µl in total, containing equal volumes of reservoir and protein solution. Crystals obtained from the initial screening were further optimized to obtain crystals suitable for X-ray data collection.

Since protein structures possessing significant sequence homology with human liver regucalcin are unknown, molecular replacement cannot be used for structure determination. Various heavy-atom derivatives were prepared by the quick-soak method (Sun *et al.*, 2002; Sun & Radaev, 2002) and tested for diffraction.

2.3. Data collection

Single crystals of both native protein and potential derivatives were transferred into Paratone-N (Hampton Research) and immediately flash-cooled in an N_2 stream at 100 K. Diffraction data were collected from native crystals using an R-AXIS V imaging-plate detector (Rigaku) at beamline BL32B2, SPring-8. Data collection from potential derivatives was performed using an R-AXIS IV^{++} imaging-plate detector (Rigaku) and Cu $K\alpha$ radiation from a RU-200 rotating-anode X-ray generator (Rigaku) operating at 50 kV and 100 mA. All data sets were processed and scaled with the program *CrystalClear* (Rigaku). Isomorphous difference and anomalous difference Patterson maps were calculated using the program *FFT* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

Table 1
Diffraction data and processing statistics.

Values in parentheses are for the outermost resolution shell.

	Native	Pb derivative
Source	Synchrotron	Cu $K\alpha$
Detector	R-AXIS V	R-AXIS IV^{++}
Wavelength (Å)	1.000	1.545
Resolution range (Å)	40.58–2.80 (2.90–2.80)	47.00–3.60 (3.73–3.60)
Space group	$P2_1$	$P2_1$
Unit-cell parameters		
a (Å)	64.87	64.31
b (Å)	52.52	53.13
c (Å)	86.38	85.68
β (°)	99.86	99.77
No. measured reflections	58404	28869
No. unique reflections	13697	6660
Completeness (%)	98.1 (96.0)	98.2 (95.3)
R_{sym} (%)	12.5 (23.4)	15.4 (30.3)
Mean $I/\sigma(I)$	5.3 (2.8)	6.1 (3.3)
Crystal mosaicity (°)	0.71	0.80

3. Results and discussion

A crystal was obtained from Crystal Screen I condition No. 22 during initial screening, but was not reproducible. The issue was settled by adding calcium ions to the reservoir solution. Finally, plate-shaped crystals were grown reproducibly from a reservoir consisting of 30–34%(*w*/*v*) PEG 4000, 0.2 *M* $Na(CH_3COO)_2$, 5 m*M* $CaCl_2$ and 0.1 *M* Tris–HCl pH 8.0. The drops contained equal volumes of protein and reservoir solution and were equilibrated against 1 ml reservoir solution at 293 K. The crystals appeared within a week and reached dimensions of up to 0.30 × 0.10 × 0.03 mm after two weeks (Fig. 1). The native crystal diffracted to 2.8 Å resolution. Based upon the presence

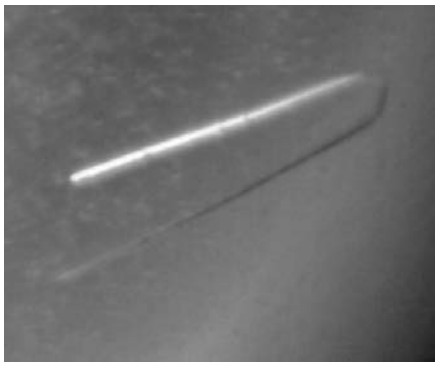

Figure 1
Crystal of human liver regucalcin.

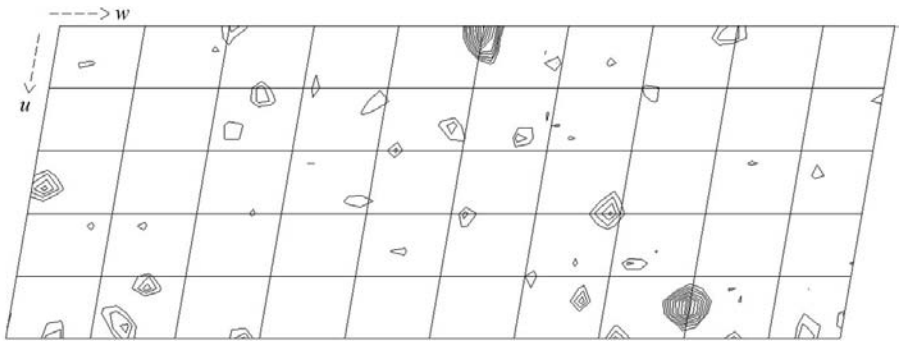

Figure 2
Isomorphous difference Patterson map. The Pb peaks are shown in the v = 0.5 Harker section.

of two molecules per asymmetric unit, the calculated Matthews coefficient is 2.2 Å^3 Da^{-1} (Matthews, 1968), corresponding to a solvent content of 43.8%.

To prepare heavy-atom derivatives, the quick-soak method (Sun *et al.*, 2002; Sun & Radaev, 2002) was used. Soaking times and heavy-atom reagent concentrations were varied from 5 to 10 min and 5 to 15 m*M*, respectively, to minimize damage to the crystals. After trials with several types of heavy-atom reagent, data obtained from a crystal soaked in 10 m*M* $Pb(CH_3COO)_2$ for 10 min showed strong peaks in an isomorphous difference Patterson map (Fig. 2). Two consistent heavy-atom positions were determined from the Patterson map (position 1, 0.2552, 0, 0.0982; position 2, 0.9342, 0.2540, 0.2477). Data-collection and processing statistics for both native and the Pb derivative are presented in Table 1. The high *R* value of both data sets might be because of the use of a cryoprotectant and/or cooling method that is not optimal for the crystals, resulting in an increase in crystal mosaicity (Table 1). Other cryoprotectants and cooling methods are therefore being investigated and a MAD experiment using the Pb derivative will be performed with the use of a synchrotron X-ray source.

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