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Crystallization and preliminary X-ray crystallographic studies of the D2 region of the skeletal muscle ryanodine receptor

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The N-terminal portion (amino acids 1303–1367) of the type 1 ryanodine receptor D2 region is thought to be critical for excitation–contraction coupling in skeletal muscle. A segment of the D2 region (amino acids 1317–1355) was expressed as a glutathione S-transferase fusion protein (GST-D2) and then crystallized at room temperature using ammonium sulfate as precipitant. Using a newly developed cryo-soaking method, complete native data sets were measured to a resolution of 2.2 Å using synchrotron radiation. The crystal was found to be hexagonal, belonging to space group $P6_322$, with unit-cell parameters $a = b = 116.1$, $c = 77.9$ Å.

1. Introduction

Action potentials initiated at the surface of skeletal muscle cells are propagated into the cell *via* the transverse tubular membrane system (T-tubule). The mechanism by which excitation at the T-tubule triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) and muscle contraction (excitation–contraction coupling, E–C coupling) is not completely understood. However, it is known that ryanodine receptors (RyRs), which are a family of Ca^{2+} channels mediating Ca^{2+} release from SR, directly communicate with voltage-sensing dihydropyridine receptors in the T-tubules of skeletal muscle (Rios & Brum, 1987; Beam *et al.*, 1986; Tanabe *et al.*, 1988; Takeshima *et al.*, 1994).

Three subtypes of RyR (RyR1, RyR2 and RyR3) have been characterized thus far (McPherson & Campbell, 1993; Meissner, 1994; Sutko & Airey, 1996) and of these, RyR1 is involved in skeletal muscle E–C coupling (Rios & Brum, 1987). Although the overall amino-acid sequence identity among RyR subtypes is 67–70%, there are several regions where the amino-acid sequences significantly diverge (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990; Nakai *et al.*, 1990; Otsu *et al.*, 1990; Hakamata *et al.*, 1992). These regions are referred to as D1, D2 and D3 (Sorrentino & Volpe, 1993), and in skeletal muscle, RyR1 D2 (amino acids 1303–1406) is thought to be essential for E–C coupling; in particular, the N-terminal region (amino acids 1303–1367) appears to play a critical role (Yamazawa *et al.*, 1997). Furthermore, the segment spanning amino acids 1317–1355 contains a substantial number of charged residues which would be expected to contribute to the formation of secondary structure. To gain additional information about the structure of that segment, we overexpressed it as a GST fusion protein (GST-D2), purified and crystallized it, and subjected it to X-ray crystallography. In the process, we developed a novel cryo-soaking method which substantially improves diffraction quality.

2. Materials and methods

2.1. Cloning and protein purification

mRNA was prepared from rabbit fast-twitch skeletal muscle using the Micro Fast Track kit (Invitrogen). cDNA was prepared using M-MuLV (Moloney Murine Leukemia Virus) reverse transcriptase. The DNA for the D2 region of RyR1 (amino acids 1317–1355) was amplified by the polymerase chain reaction

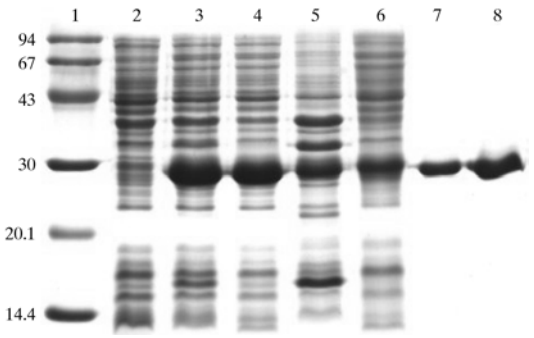


Figure 1
GST-D2 overexpression and purification revealed by 15% SDS–PAGE; the gel was stained with Coomassie brilliant blue. Lanes are as follows: lane 1, Pharmacia low molecular weight protein markers (molecular weights are shown in kDa); lane 2, extract from non-induced cells; lane 3, extract from cells induced with 0.4 m*M* isopropyl β-D-thiogalactopyranoside; lane 4, cell supernatant; lane 5, cell precipitate; lane 6, unbound fraction from the glutathione-Sepharose 4B column; lane 7, pooled eluate containing the bound fraction from the glutathione–Sepharose 4B column; lane 8, concentrated eluate from the Superdex-200 column.

Table 1
Summary of the GST-D2 data sets obtained at cryogenic temperatures.

Values in parentheses are for the highest resolution shell.

Data set	R-AXIS IV	PF (BL-6A)
Space group	$P6_322$	$P6_322$
Unit-cell dimensions (Å)	$a = b = 116.4$, $c = 77.6$	$a = b = 116.1$, $c = 77.9$
Resolution of data (Å)	3.0	2.2
Number of data collected	98721	112574
Number of unique data	6561	16398
Completeness	97.6 (99.7)	99.1 (99.6)
$R_{\rm merge}$†	6.7	7.6
Mean $\langle I/\sigma(I)\rangle$	18.1 (4.9)	11.3 (2.8)

† $R_{\rm merge}(I) = \sum_h \sum_i |I_i - I| / \sum_h \sum_i I$, where I is the mean intensity of i reflections h.

(PCR), subcloned between the *Eco*RI and *Xho*I sites of the expression vector pGEX4T-1 (Pharmacia Biotech) and transformed into *Escherichia coli* strain BL21(DE3). GST-D2 was induced by 0.4 m*M* IPTG (isopropyl β-D-thiogalactopyranoside) for 16 h at 310 K. Bacterial cells were lysed using a French press. Lysates were cleared by centrifugation and then applied to a glutathione-Sepharose 4B affinity chromatography column. The GST-D2-containing eluate was further purified by gel filtration on a Superdex 200 column. The fractions containing GST-D2 were collected and concentrated by ultrafiltration (Amicon Centriprep 10).

2.2. Crystallization and data collection

GST-D2 was crystallized at room temperature (294 ± 1 K) using the hanging-drop vapour-diffusion method. Crystals were grown on a siliconized cover slip by equilibrating a mixture containing 2 µl of protein solution [14 mg ml^{-1} protein in 20 m*M* HEPES pH 7.2, 150 m*M* KCl, 10 m*M* dithiothreitol, 10 m*M* GSH (tripeptide glutathione, γ-Glu-Cys-Gly)] and 2 µl reservoir solution (1.8–1.9 *M* ammonium sulfate, 0.1 *M* sodium citrate pH 5.1) against 0.5 ml of reservoir solution. Two sets of native data were then collected.

The first set of native data was collected on an R-AXIS IV image-plate system attached to a Rigaku rotating-anode generator (RU-300) providing Cu $K\alpha$ radiation and running at 50 kV and 90 mA with a 0.3 mm focus cup. For the cryogenic experiments, the cryoprotectant was determined to be mother liquor plus 30% glycerol. Successful flash-freezing was achieved when the crystals were sequentially transferred in three steps to cryosolvents containing between 10 and 30% glycerol and were allowed to equilibrate for 10 min at each step.

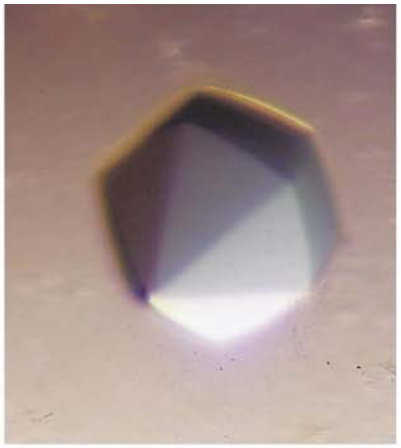
The second set of native data was collected using a Weissenberg camera for macromolecular crystallography at the BL-6A experimental station of the Photon Factory, Tsukuba, Japan (Sakabe, 1991). The wavelength of the synchrotron X-rays was 1.000 Å and a 0.1 mm collimator was used. Fuji image plates (type BAI II, 20 × 40 cm) were placed 430 mm from the crystal. The oscillation range per image plate was 0–4.0° at a rate of 2.0° s^{-1}. The overlap between contiguous image plates was 0.5°. The diffraction patterns recorded on the image plates were digitized using a Fuji BA100 scanner.

To improve diffraction quality, the cryo-soaking method was modified for the second native data set. In this case, crystals were sequentially transferred to cryosolvents containing 5–25% glycerol in five steps (5% glycerol concentration intervals), equilibrating for 30 s at each step; after the final transfer crystals were equilibrated for 30 min. The cryosolvent containing the crystals was then air-dried until ammonium sulfate crystals began to appear (about 20 min at room temperature). X-ray diffraction data were collected in a nitrogen-gas stream at 110 K (Oxford Cryosystems). Both native data sets were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1996).

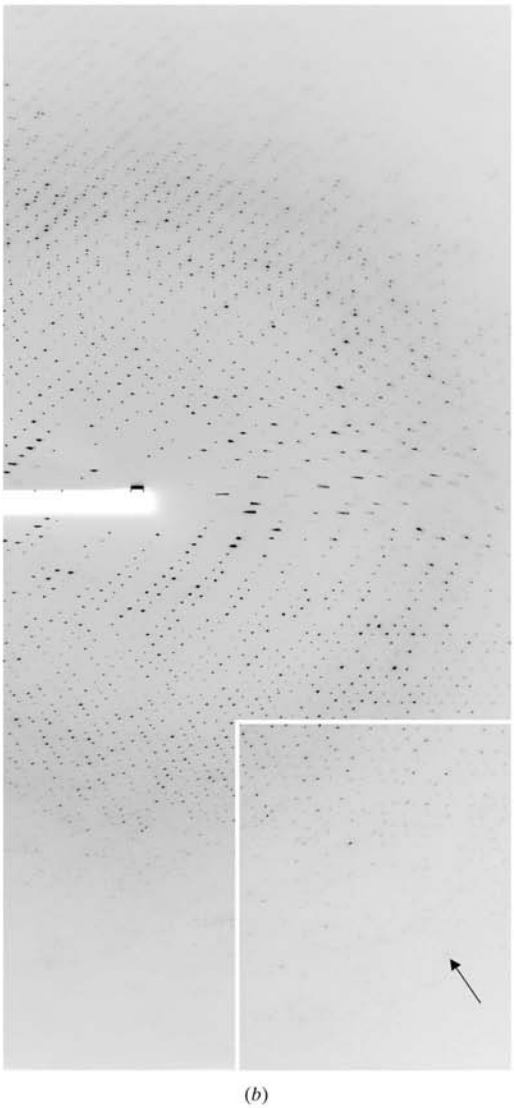
3. Results and discussion

Fig. 1 shows the results of SDS–PAGE analysis of a GST-D2 preparation which was sufficiently pure to be used in crystallization trials. Hexagonal crystals (0.5 × 0.5 × 0.5 mm) were obtained from this preparation within 3 d. The statistics of the native data sets obtained from the X-ray crystallographic analysis are summarized in Table 1.

The first native data set was collected using Cu $K\alpha$ X-rays and the R-AXIS IV system. The diffraction pattern from a flash-frozen GST-D2 crystal was observed to a limit of 3.0 Å on the image plate of the detector. The space group was determined to be $P6_322$ using *DENZO* autoindexing (Otwinowski & Minor, 1996) and



(*a*)



(*b*)

Figure 2
(*a*) GST-D2 crystal grown for 3 d using 1.85 *M* ammonium sulfate as a precipitating agent (pH 5.1). Its approximate dimensions are 0.5 × 0.5 × 0.5 mm. (*b*) 4° Weissenberg image obtained at the Photon Factory (BL-6A) using a Weissenberg camera at a distance of 430 mm. The X-ray wavelength was 1.000 Å and the flash-frozen crystal diffracted to 2.2 Å resolution. The arrow in the figure indicates a diffraction of 2.3 Å resolution.

the molecular-replacement method using the atomic coordinates of a 2.4 Å *Schistosoma japonica* GST crystal structure (Sja GST; McTigue *et al.*, 1995). The unit-cell parameters at cryogenic temperature (110 K) were $a = b = 116.4$, $c = 77.6$ Å. In the highest resolution shell, the data were 99.7% complete and $I/\sigma(I) = 4.9$. The data set consists of 98721 total measurements of 6561 unique reflections ($R_{\rm merge}$ is 6.7%). The unit-cell volume was 910 358 Å^3 and the molecular weight of each subunit (274 amino acids) was 31569 Da. The presence of one molecule in the asymmetric unit yielded a unit-cell volume per protein mass (V_m) of 2.40 Å^3 Da^{-1} and a solvent content of 48.8% by volume; these values are well within the range of previously observed protein crystals (Matthews, 1968).

The second native data set was collected with synchrotron X-rays using a Weissenberg camera at the Photon Factory. A crystal soaked following the same protocol as was used for R-AXIS data collection diffracted to about 3 Å, the same as with Cu $K\alpha$ X-rays. However, using our new method for cryo-soaking, the diffraction limit improved substantially. Indeed, the diffraction pattern from a flash-frozen GST-D2 crystal was observed to 2.2 Å on a Fuji image plate (Fig. 2). The space group was determined to be hexagonal $P6_322$, with unit-cell parameters were $a = b = 116.1$, $c = 77.9$ Å. The collected data consisted of 112574 measurements of 16398 unique reflections. The data in the highest resolution shell was 99.6% complete and $I/\sigma(I) = 2.8$. The unit-cell volume was 909547 Å^3 and the molecular weight of the subunit was 31569 Da. The presence of one molecule in the asymmetric unit yielded a V_m of 2.40 Å^3 Da^{-1} and a solvent content of 48.8% by volume. To determine the structure, a combination of the molecular-replacement method using Sja GST coordinates (McTigue *et al.*, 1995) and multiple isomorphous replacement methods is currently under way in our laboratory.

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