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Crystallization and preliminary X-ray analysis of the Ca^{2+}-bound C-terminal lobe of troponin C in complex with a troponin I-derived peptide fragment from Akazara scallop

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Troponin C (TnC) is the Ca^{2+}-binding component of troponin and triggers muscle contraction. TnC of the invertebrate Akazara scallop can bind only one Ca^{2+} at the C-terminal EF-hand motif. Recombinant TnC was expressed in *Escherichia coli*, purified, complexed with a 24-residue synthetic peptide derived from scallop troponin I (TnI) and crystallized. The crystals diffracted X-rays to 1.80 Å resolution and belonged to space group $P2_12_12_1$, with unit-cell parameters a = 32.1, b = 42.2, c = 60.0 Å. The asymmetric unit was assumed to contain one molecular complex of the Akazara scallop TnC C-lobe and TnI fragment, with a Matthews coefficient of 1.83 $Å^3$ Da^{-1} and a solvent content of 33.0%.

1. Introduction

Striated muscle contraction is triggered by Ca^{2+} binding to troponin (Tn), which is composed of three components: troponin C (TnC; the Ca^{2+}-binding component), troponin I (TnI; the component that inhibits the ATPase activity of actomyosin) and troponin T (TnT; the tropomyosin-binding component) (Ohtsuki *et al.*, 1986; Farah & Reinach, 1995). Vertebrate TnCs have three to four Ca^{2+}-binding sites: one or two regulatory low-affinity site(s) in the N-terminal lobe (N-lobe) and two high-affinity sites in the C-terminal lobe (C-lobe) (Zot & Potter, 1987). Crystal structures of vertebrate TnCs have been determined (Herzberg & James, 1985; Sundaralingam *et al.*, 1985). Moreover, numerous structural studies have been performed on vertebrate skeletal muscle and cardiac Tns (Gagné *et al.*, 1995; Takeda *et al.*, 2003; Vinogradova *et al.*, 2005) and the molecular mechanism of Ca^{2+} regulation of vertebrate muscle contraction has been elucidated. In contrast, scallop TnC, an invertebrate TnC, can only bind one Ca^{2+} ion at the C-terminal EF-hand motif (site IV) in the C-lobe (Ojima & Nishita, 1986; Ojima *et al.*, 2000). In order to elucidate the molecular mechanism of the regulation of scallop striated adductor muscle contraction, we focused on the three-dimensional structural analysis of the C-lobe of Akazara scallop TnC in complex with a TnI-fragment peptide. Previously, we reported an NMR structural study of the C-lobe of Akazara scallop TnC in the presence of a TnI-fragment peptide, in which we could determine the structure of the TnC C-lobe but not that of the TnI peptide (Yumoto *et al.*, 2003). Therefore, we attempted cocrystallization of the TnC C-lobe and TnI peptide in order to solve the structure of the complex by X-ray crystallography. Here, we report the crystallization and preliminary X-ray analysis of a binary complex of the scallop TnC C-lobe and a 24-residue TnI peptide.

2. Materials and methods

2.1. Protein expression and purification

The cDNA encoding the Akazara scallop TnC C-lobe (8.5 kDa; **M**EDLDERELKEAFRVLDKEKKGVIKVDVLRWILKSLGDELT-EDEIENMIAETDTDGSGTVDYEEFKCLMMSSDA, corresponding to the C-terminal 73 residues of 152 with an additional N-terminal methionine residue which is shown in bold) was amplified by PCR

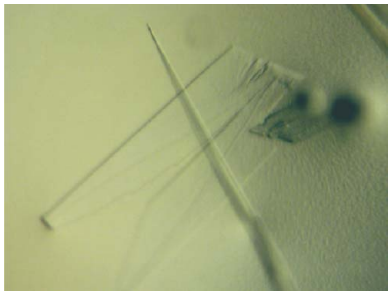

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	PF-AR NW12
Wavelength (Å)	1.000
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 32.1, b = 42.2, c = 60.0
Resolution range (Å)	50.0–1.80 (1.86–1.80)
Observed reflections	153452
Unique reflections	8036
Completeness (%)	99.5 (97.8)
R_{sym}† (%)	5.3 (26.6)
$\langle I\rangle/\langle\sigma(I)\rangle$	12.2 (5.5)

† $R_{sym} = \sum_{\mathbf{h}}\sum_{l}|I_{\mathbf{h}l} - \langle I_{\mathbf{h}}\rangle|/\sum_{\mathbf{h}}\sum_{l}\langle I_{\mathbf{h}}\rangle$, where $I_{\mathbf{h}l}$ is the lth observation of reflection **h** and $\langle I_{\mathbf{h}}\rangle$ is the weighted average intensity for all observations l of reflection **h**.

using the cDNA clone of Akazara scallop TnC (Ojima *et al.*, 1994, 1997) as the template DNA and primers containing *Nco*I and *Bam*HI sites. The amplified gene was inserted into the *Nco*I/*Bam*HI-digested expression vector pET-16b (Novagen). The recombinant plasmid was transformed into *Esherichia coli* strain BL21(DE3) (Novagen). The *E. coli* cells were grown in 2 l Luria–Bertani medium supplemented with 100 µg ml^{-1} ampicillin in a 5 l Erlenmeyer flask at 310 K until the OD_{600} reached 0.8. Expression of the TnC C-lobe was induced with 1.0 m*M* (final concentration) isopropyl β-D-thiogalactopyranoside (IPTG) and incubation continued for a further 4 h at 310 K. The cells were collected by centrifugation at 5180*g* for 10 min at 277 K, resuspended in a buffer containing 20 m*M* Tris–HCl pH 8.0, 0.1 m*M* EDTA and 1 m*M* DTT and then disrupted by sonication. After centrifugation at 48 000*g* for 30 min at 277 K, the supernatant was incubated with 30%(*w*/*v*) (final concentration) ammonium sulfate for 0.5 h at 293 K. After another round of centrifugation, the supernatant, which contained the Akazara scallop TnC C-lobe, was treated with 3%(*v*/*v*) (final concentration) TCA to precipitate the TnC C-lobe without Ca^{2+} (apo form). In general, EF-hand proteins bind not only Ca^{2+} but also other cations. TCA treatment has been used previously to remove bound cations (Ca^{2+} and other cations) from EF-hand proteins (Yagi *et al.*, 1982; Tanokura & Yamada, 1984; Yumoto *et al.*, 2001; Nara *et al.*, 2004). We therefore initially prepared an apo form of our protein by TCA precipitation and then subsequently prepared the Ca^{2+}-bound form by adding Ca^{2+} to the apo form (see below). The TCA-precipiated apo TnC C-lobe was resolubilized by adding 1 *M* Tris base and then dialyzed against 20 m*M* Tris–HCl pH 8.0, 0.1 m*M* EDTA and 1 m*M* DTT. After centrifugation at 48 000*g* for 30 min at 277 K, the supernatant was loaded onto a DEAE-Toyopearl 650M (Tosoh) column. Stepwise elution was carried out with 20 m*M* Tris–HCl pH 8.0, 0.1 m*M* EDTA and 1 m*M* DTT supplemented with 100, 200, 300, 400 and 500 m*M* NaCl. The fractions thus obtained were analyzed by SDS–PAGE and the TnC C-lobe-containing fractions were collected. Apo TnC C-lobe was concentrated using VivaSpin (3000 Da molecular-weight cutoff, Vivascience) and loaded onto a Superdex 75 10/30 column (GE Healthcare) equilibrated with 20 m*M* Tris–HCl pH 8.0, 0.1 m*M* EDTA, 1 m*M* DTT and 150 m*M* NaCl. The eluted fractions were analyzed by SDS–PAGE to assess the purity of the protein. Selenomethionine-labelled TnC C-lobe was produced with the same plasmid using M9 medium supplemented with 100 µg ml^{-1} ampicillin, selenomethionine and trace elements (Cai *et al.*, 1998). For the purification of the SeMet-labelled TnC C-lobe, 10 m*M* DTT was added in all purification steps.

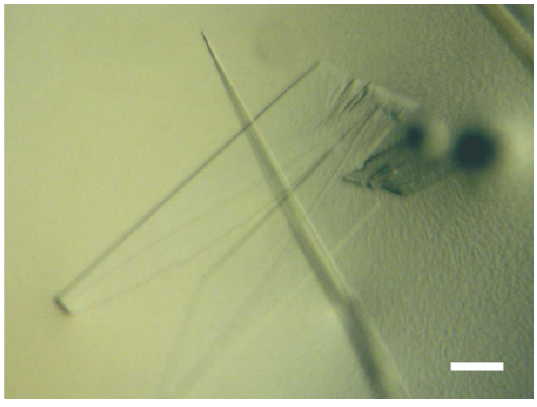

Figure 1
Crystals of the C-lobe of Akazara scallop TnC complexed with the TnI-peptide fragment. The scale bar corresponds to 100 µm.

The chemically synthesized TnI-fragment peptide (2.7 kDa, NH_2-GLSPEKKKMLKKLIMQKAAEDLAN-COOH, 24 residues) was purchased from Operon Biotechnologies (Tokyo, Japan).

Apo TnC C-lobe, TnI-fragment peptide and $CaCl_2$ solutions were mixed to give a solution containing 1.2 m*M* TnC C-lobe, 1.8 m*M* TnI-fragment peptide, 10 m*M* $CaCl_2$, 20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl and 2 m*M* DTT. The mixture was first incubated at 323 K for 1 h and then at 293 K for 2 h to facilitate complex formation. The sample was filtered using a 0.2 µm filter and was loaded onto a Superdex 75 10/30 column equilibrated with 10 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 10 m*M* $CaCl_2$ and 2 m*M* DTT. The complex eluted at the expected molecular weight of 11.1 kDa and was concentrated to 3.6 mg ml^{-1} in a buffer consisting of 10 m*M* Tris–HCl pH 8.0, 10 m*M* $CaCl_2$ and 10 m*M* DTT using Vivaspin (3000 Da molecular-weight cutoff). In the case of the SeMet-labelled TnC C-lobe and TnI-peptide complex, 10 m*M* DTT was included in the complex-formation and purification steps. The SeMet-labelled complex was concentrated to 7.5 mg ml^{-1} in a buffer consisting of 5 m*M* Tris–HCl pH 8.0, 10 m*M* $CaCl_2$ and 10 m*M* DTT using Vivaspin (3000 Da molecular-weight cutoff).

2.2. Crystallization

The sparse-matrix screening kit Crystal Screen HT (Hampton Research) was used for initial crystallization trials in conjunction with the sitting-drop vapour-diffusion method. Drops made manually by mixing 0.8 µl protein solution and 0.8 µl reservoir solution were equilibrated against 70 µl reservoir solution in Intelli-Plates (Art Robbins Enterprises) and incubated at 293 K.

In the case of SeMet-labelled TnC C-lobe complexed with unlabelled TnI peptide, drops made manually by mixing 1.5 µl protein solution and 1.5 µl reservoir solution were equilibrated against 500 µl reservoir solution in a 24-well plate using the sitting-drop vapour-diffusion method (Hampton Research) and incubated at 293 K.

2.3. X-ray data collection

X-ray diffraction experiments were performed at beamline NW12 of the Photon Factory-Advanced Ring (PF-AR), Tsukuba, Japan. The crystals were transferred into a cryoprotectant solution containing 0.08 *M* sodium acetate pH 4.4, 0.16 *M* ammonium acetate, 29.6% PEG 4000 and 20% ethylene glycol for approximately 10–30 s. The crystals were then mounted in nylon loops (Hampton Research) and flash-cooled in a nitrogen stream at 95 K. Diffraction data were collected in 1.0° oscillation steps using a Quantum 210 CCD X-ray detector (Area Detector Systems Corporation).

3. Results and discussion

Single crystals of unlabelled complex were obtained in one day using a reservoir solution consisting of 30% PEG 4000, 0.1 *M* sodium acetate pH 4.6 and 0.2 *M* ammonium acetate. Subsequently, the pH and precipitant concentration of the reservoir solution were optimized. The optimized reservoir solution consisted of 37% PEG 4000, 0.1 *M* sodium acetate pH 4.0 and 0.2 *M* ammonium acetate. The crystals obtained after optimization are shown in Fig. 1 and had typical dimensions of 600 × 100 × 10 µm. The best crystal diffracted X-rays to a resolution of 1.8 Å. The X-ray diffraction data set was processed and scaled using *HKL*-2000 (Otwinowski & Minor, 1997). The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 32.1, b = 42.2, c = 60.0 Å. The data-collection statistics for the best crystal are shown in Table 1. The asymmetric unit was assumed to contain one molecular complex of the TnC C-lobe and the TnI fragment; the Matthews coefficient was 1.83 $Å^3\,Da^{-1}$ (Matthews, 1968) and the solvent content was 33.0%.

Single crystals of SeMet TnC C-lobe complexed with unlabelled TnI fragment were obtained in one day using a reservoir solution consisting of 35% PEG 4000, 0.1 *M* sodium acetate pH 4.0 and 0.2 *M* ammonium acetate. Structure solution and refinement by the MAD method using SeMet-labelled TnC C-lobe complexed with an unlabelled TnI-peptide fragment are now in progress.

The synchrotron-radiation experiments were performed at NW12 at the Photon Factory Advanced Ring (PF-AR, Tsukuba, Japan) with the approval of the Photon Factory, KEK (Proposal No. 2006S2-006). We would like to thank the beamline staff at the Photon Factory for their kind help with data collection. This work was supported in part by the National Project on Protein Structural and Functional Analyses of the Ministry of Education, Culture, Sports, Science and Technology of Japan and by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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