Fumiaki Yumoto,^{a,b} Koji Nagata,^a
Yumiko Miyauchi,^a Takao
Ojima,^c Hiroyuki Tanaka,^c
Kiyoyoshi Nishita,^c Iwao
Ohtsuki^b and Masaru Tanokura^{a*}

^aDepartment of Applied Biological Chemistry,
Graduate School of Agricultural and Life
Sciences, Bunkyo-ku, Tokyo 113-8657, Japan,

^bDepartment of Physiology II,
The Jikei University School of Medicine,
3-19-18 Nishi-shinbashi, Minato-ku,
Tokyo 105-8461, Japan, and ^cLaboratory of
Biochemistry and Biotechnology, Graduate
School of Fisheries Sciences, Hokkaido
University, Hakodate, Hokkaido 041-8611,
Japan

Correspondence e-mail:
amtanok@mail.ecc.u-tokyo.ac.jp

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Crystallization and preliminary X-ray analysis of the Ca²⁺-bound C-terminal lobe of troponin C in complex with a troponin I-derived peptide fragment from Akazara scallop

Troponin C (TnC) is the Ca²⁺-binding component of troponin and triggers muscle contraction. TnC of the invertebrate Akazara scallop can bind only one Ca²⁺ 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 *P*2₁2₁2₁, 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 Å³ Da⁻¹ and a solvent content of 33.0%.

1. Introduction

Striated muscle contraction is triggered by Ca²⁺ binding to troponin (Tn), which is composed of three components: troponin C (TnC; the Ca²⁺-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 Ca²⁺-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 Ca²⁺ regulation of vertebrate muscle contraction has been elucidated. In contrast, scallop TnC, an invertebrate TnC, can only bind one Ca²⁺ 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; **MED**LDERELKEAFRVLDKEKKGVIKVDVLRWILKSLGDELT-**EDEIENMIAETD**TDGSGTVDYEEFKCLMMSSDA, 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)
$R_{\text{sym}}^{\dagger}$ (%)	5.3 (26.6)
$\langle I \rangle / \langle \sigma(I) \rangle$	12.2 (5.5)

$\dagger R_{\text{sym}} = \sum_h \sum_l |I_{hl} - \langle I_h \rangle| / \sum_h \sum_l \langle I_h \rangle$, where I_{hl} is the l th observation of reflection h and $\langle I_h \rangle$ 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 *Escherichia 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 OD₆₀₀ reached 0.8. Expression of the TnC C-lobe was induced with 1.0 mM (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 5180g for 10 min at 277 K, resuspended in a buffer containing 20 mM Tris–HCl pH 8.0, 0.1 mM EDTA and 1 mM DTT and then disrupted by sonication. After centrifugation at 48 000g 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 Ca²⁺ (apo form). In general, EF-hand proteins bind not only Ca²⁺ but also other cations. TCA treatment has been used previously to remove bound cations (Ca²⁺ 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 Ca²⁺-bound form by adding Ca²⁺ to the apo form (see below). The TCA-precipitated apo TnC C-lobe was resolubilized by adding 1 M Tris base and then dialyzed against 20 mM Tris–HCl pH 8.0, 0.1 mM EDTA and 1 mM DTT. After centrifugation at 48 000g for 30 min at 277 K, the supernatant was loaded onto a

DEAE-Toyopearl 650M (Tosoh) column. Stepwise elution was carried out with 20 mM Tris–HCl pH 8.0, 0.1 mM EDTA and 1 mM DTT supplemented with 100, 200, 300, 400 and 500 mM 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 mM Tris–HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT and 150 mM 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 mM DTT was added in all purification steps.

The chemically synthesized TnI-fragment peptide (2.7 kDa, NH₂-GLSPEKKKMLKKLIMQKAAEDLAN-COOH, 24 residues) was purchased from Operon Biotechnologies (Tokyo, Japan).

Apo TnC C-lobe, TnI-fragment peptide and CaCl₂ solutions were mixed to give a solution containing 1.2 mM TnC C-lobe, 1.8 mM TnI-fragment peptide, 10 mM CaCl₂, 20 mM Tris–HCl pH 8.0, 200 mM NaCl and 2 mM 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 mM Tris–HCl pH 8.0, 200 mM NaCl, 10 mM CaCl₂ and 2 mM 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 mM Tris–HCl pH 8.0, 10 mM CaCl₂ and 10 mM DTT using VivaSpin (3000 Da molecular-weight cutoff). In the case of the SeMet-labelled TnC C-lobe and TnI-peptide complex, 10 mM 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 mM Tris–HCl pH 8.0, 10 mM CaCl₂ and 10 mM 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).

Figure 1

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

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 $600 \times 100 \times 10 \mu\text{m}$. 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 $1.83 \text{ Å}^3 \text{ Da}^{-1}$ (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.

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