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**FOR THE RECORD**

# Production, crystallization, and preliminary X-ray analysis of rabbit skeletal muscle troponin complex consisting of troponin C and fragment (1–47) of troponin I

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**Abstract:** Troponin is a ternary protein complex consisting of subunits TnC, TnI, and TnT, and plays a key role in calcium regulation of the skeletal and cardiac muscle contraction. In the present study, a partial complex (CI47) was prepared from *Escherichia coli*-expressed rabbit skeletal muscle TnC and fragment 1–47 of TnI, which is obtained by chemical cleavage of an *E. coli*-expressed mutant of rabbit skeletal muscle TnI. Within the ternary troponin complex, CI47 is thought to form a core that is resistant to proteolytic digestion, and the interaction within CI47 likely maintains the integrity of the troponin complex. Complex CI47 was crystallized in the presence of sodium citrate. The addition of trehalose improved the diffraction pattern of the crystals substantially. The crystal lattice belongs to the space group $P3_{1(2)}21$, with unit cell dimensions $a = b = 48.2$ Å, $c = 162$ Å. The asymmetric unit presumably contains one CI47 complex. Soaking with *p*-chloromercuribenzenesulfonate (PCMBS) resulted in loss of isomorphism, but enhanced the quality of the crystals. The crystals diffracted up to 2.3 Å resolution, with completeness of 91% and $R_{merge} = 6.4\%$. The crystals of PCMBS-derivative should be suitable for X-ray studies using the multiple-wavelength anomalous diffraction technique. This is the first step for elucidating the structure of the full troponin complex.

**Keywords:** chemical cleavage; crystallization; mass spectrometry; skeletal muscle regulation; troponin; X-ray crystallography

In the skeletal and the cardiac muscles of vertebrates and other phyla, troponin and tropomyosin are associated with actin filaments, constituting the thin filament that plays important roles in contraction and regulation. Muscle contraction is regulated by concentrations of $Ca^{2+}$, which binds to troponin (Ebashi & Endo, 1968). $Ca^{2+}$ binding to troponin shifts the equilibrium between an on state and an off state of the thin filament complex (cf. Lehrer, 1994). Since its discovery as the first example of a calcium concentration-dependent regulatory mechanism in biological systems, the troponin–tropomyosin system has been studied intensively. However, the molecular mechanism of calcium regulation at atomic level remains obscure because the 3D structure of the system is still unknown.

Troponin is a ternary complex of subunits TnC, TnI, and TnT. To date, the atomic structures of only TnC in the absence (Herzberg & James, 1985; Sundaralingam et al., 1985; Slupsky & Sykes, 1995) and in the presence (Slupsky & Sykes, 1995) of $Ca^{2+}$ in its regulatory sites have been reported. No crystal of the troponin complex has been produced that is suitable for X-ray diffraction studies at an atomic level.

We have so far obtained several crystal forms of various types of the troponin complexes (Takeda, 1997), although the quality of most of the crystals should be improved. Our basic strategies included the following. (1) Recombinant proteins are expressed in *Escherichia coli*, precluding heterogeneous nature of the proteins prepared from muscle, especially TnT, which contains many iso-

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*Abbreviations:* TnC, TnI, and TnT, troponin C, I, and T, respectively; CI47, troponin complex consisting of TnC and amino-terminal fragment (1–47) of TnI; PCMBS, *p*-chloromercuribenzenesulfonate; ESRF, European Synchrotron Radiation Facility; MAD, multiple-wavelength anomalous diffraction; DTT, dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; $TnI_{1-47}$, N-terminal fragment of TnI residues 1–47; NTCB, 2-nitro-5-thiocyanobenzoic acid; MIR, multiple isomorphous replacement.

forms (Smillie et al., 1988; Fujita et al., 1991). (2) Cores that are resistant to proteolytic enzymes are produced (S. Takeda, T. Kobayashi, H. Taniguchi, H. Hayashi, Y. Maéda, in prep.) or partial complexes are formed in order to minimize the conformational heterogeneity (Takeda, 1997; S. Takeda, T. Kobayashi, H. Taniguchi, H. Hayashi, Y. Maéda, in prep.).

In the present study, crystals that are suitable for X-ray diffraction studies were obtained for a partial complex (CI47) of TnC and the N-terminal fragment of TnI residues 1–47. N-terminal fragments of TnI have been known to form a binary complex with TnC (Ngai & Hodges, 1992; Syska et al., 1976), which is resistant to proteolytic digestion (Takeda, 1997; S. Takeda, T. Kobayashi, H. Taniguchi, H. Hayashi, Y. Maéda, in prep.). TnI binds to TnC in an antiparallel manner so that the N-terminal fragment of TnI interacts with the C-terminal half of TnC (Farah et al., 1994; Kobayashi et al., 1994; Krudy et al., 1994). The affinity is higher if two $Ca^{2+}$–$Mg^{2+}$ binding sites are occupied in the C-terminal half of TnC (Sheng et al., 1992; Farah et al., 1994). This suggests that the major role of the interaction between TnC and the N-terminal fragment of TnI is to maintain the integrity of the troponin complex. The atomic structure of CI47 is crucial to understanding the mechanism of formation of the troponin complex.

**Results and discussion:** In the present study, $TnI_{1-47}$ was prepared by a combination of two techniques, cysteine cleavage and site-directed mutagenesis. It is difficult to prepare peptides of such length using either peptide synthesis or direct expression in *E. coli*. On the other hand, cysteine cleavage applied to the wild-type TnI, which has three cysteine residues at 48, 63, and 133, would give rise to peptides that cannot be separated from each other completely.

Although the purification procedures were optimized, the $TnI_{1-47}$ preparation was found to be a mixture of two species, fragment 1–47 and a larger species at roughly an equimolar ratio as revealed by mass spectrometry; the observed mass and the mass deduced from fragment 1–47 were 5,534.7 versus 5,533.5, whereas the other was 5,635.7, which is close to 5,636.8 expected from fragment 1–48. The nature of the second species remains to be identified.

Mass spectrometry also indicated that the *E. coil*-expressed TnC preparation contained two species at roughly an equimolar ratio, with and without an acetyl group bound to the terminal amino group of the peptide. The measured mass versus the deduced mass for the species with the acetylated N-terminus was 18,007.4 versus 18,008.0, whereas for the species without the acetyl group it was 17,964.8 versus 17,965.9. Partial N-terminal acetylation of *E. coli*-expressed proteins of mammalian origin has been confirmed (for references, see Lischwe et al., 1993).

The crystals of the CI47 complex were likely formed by the binary CI47 complex, because reversed-phase HPLC indicated that dissolved crystals contained TnC and $TnI_{1-47}$ in an equimolar ratio. Moreover, mass spectrometry showed that two species of TnC, as well as two species of $TnI_{1-47}$, coexisted in a molar ratio similar to the original preparation. In spite of heterogeneity of both TnC and $TnI_{1-47}$, the crystals were good enough and diffracted X-rays up to 2.3 Å resolution. Under the crystallization conditions, two $Ca^{2+}$–$Mg^{2+}$-binding sites in the C-terminal half of TnC are likely to be occupied. However, it is unclear whether or not the calcium-specific regulatory sites in the N-terminal half are occupied by metal ions.

Trehalose (15%) was included not only as a cryo-protective agent, but also in the aim of improving the resolution of the diffraction data (Schick & Jurnak, 1994). In fact, using a laboratory X-ray source, the resolution was extended from 3.0 Å to 2.6 Å for the crystals grown in the presence of trehalose. The addition of trehalose was associated with shrinkage of the unit lattice ($a = b =$ 49 Å, $c = 163.8$ Å changed to $a = b = 48.2$ Å, $c = 161.9$ Å). It is worth noting that PCMBS labeling resulted in further shrinkage of the lattice (7.5% of the unit cell volume), while diffraction was further improved (Table 1).

Due to the loss of isomorphism, the PCMBS derivative is not suitable for the MIR phasing. Instead, this derivative should be suitable for the MAD experiments. The data collected for three crystals of CI47 PCMBS derivative revealed that the unit cell dimensions are reproducible, and the data collection statistics are at least as good as those of the native crystals (Table 1).

**Material and methods:** *Protein expression and purification:* $TnI_{1-47}$ was obtained by a chemical cleavage (Swenson & Fredricksen, 1992) of the unique cysteine of residue 48 of a rabbit skeletal muscle TnI variant (C64A, C133S). The variant TnI was expressed and purified according to the procedures reported previously (Fujita-Becker et al., 1993; Kluwe et al., 1993). The protein at a concentration of 5 mg/mL was dissolved in a solution containing 0.2 M Tris-HCl, pH 8.0, 6 M guanidine hydrochloride, and 0.2 mM DTT. NTCB (Sigma) was added to 10-fold excess over the total sulfhydryl groups contained in the buffer and TnI. The mixture was pre-incubated for 20 min at 37 °C, then pH was increased to 9 by addition of NaOH to start the reaction, which proceeded for 6 h. The mixture of products was then applied to a gel filtration column (Sephacryl S-100, 2.5 × 100 cm, Pharmacia) equilibrated with 0.3 M NaCl, 10 mM Tris-HCl, pH 8.0. $TnI_{1-47}$, which was eluted with the same solution, was further purified by a reversed-phase column (1.0 × 30 cm, Aquapore Butyl column, Brownlee). The purified fragment was lyophilized and stored at −20 °C.

Rabbit skeletal muscle TnC was expressed in and purified from *E. coli* as described previously (Fujita-Becker et al., 1993).

$TnI_{1-47}$ was mixed with TnC in a molar excess of $TnI_{1-47}$ over TnC in the solution containing 6 M urea, 1 M NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$, 1 mM DTT. The mixture was then dialyzed consecutively against NaCl solution of 1 M, 0.7 M, 0.5 M, 0.3 M, and 0.1 M, each containing 5 mM $CaCl_2$, 20 mM Tris-HCl, pH 8.0, 1 mM DTT. After dialysis, the protein solution was clarified by centrifugation and then applied to a Q-Sepharose

**Table 1.** *Crystallographic parameters and data collection statistics of crystals of troponin CI47 complex*

| Crystals | Native | PCMBS-derivative |
|---|---|---|
| Wavelength (beam line) | 0.99 Å (ID2) | 1.0092 Å (BM14) |
| Unit cell dimensions | $a = b = 48.2$ Å | $a = b = 47.5$ Å |
| | $c = 161.9$ | $c = 153.8$ |
| Space group | $P3_{1(2)}21$ | $P3_{1(2)}21$ |
| Complex/a.u. | 1 | 1 |
| Resolution | 2.3 Å | 2.3 Å |
| Total observations | 65,317 | 77,100 |
| Unique reflections | 8,759 | 15,744 |
| Completeness | 84.8% ($\sigma = 1.0$) | 91.0% |
| $R_{merge}$ | 6.8% | 6.4% |

fast flow column equilibrated with the same solution. CI47 was eluted by a gradient of NaCl from 0.1 to 0.6 M. Fractions containing CI47 were pooled and dialyzed against 10 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$. The preparation was concentrated and used for crystallization. The complex was identified by SDS-PAGE and on a reversed-phase HPLC.

Homogeneity of the preparation was checked routinely using liquid chromatography/electrospray mass spectrometry, as described previously (Taniguchi et al., 1994).

*Crystallization:* For crystallization, the hanging-drop method was employed at 16 °C by mixing the protein solution containing 25–30 mg/mL of the CI47 complex with the reservoir solution containing 1.5 M sodium citrate, 0.1 M Tris-HCl, pH 8.0, 15% trehalose. Rhombohedral crystals grew to 0.6–1-mm long within one or two weeks. Heavy-atom derivative was produced by soaking the crystals in the reservoir solution, containing 1 mM PCMBS-Na, at 16 °C for 18 h. The crystals, after placement in the reservoir solution, were mounted on a rayon loop of a Crystal-Cap system of Hampton Research, and quickly frozen by plunging into liquid propane.

*X-ray diffraction study:* Rotation photographs were obtained either at ID2 (BL4) or at BM14 (BL19) of the ESRF (Grenoble), using an imaging plate detector of MAR-research (30-cm diameter). The crystal to detector distance was fixed to 31 cm. Exposures were made for 5 s (ID2) or 20 s (BM14) while the crystals were rotated by 2.5 degrees around the spindle axis, which was horizontal. Crystals were mounted with the *c*-axis roughly parallel to the spindle axis, and were cooled to 100 K by using a Cryostream (Oxford Cryosystems). The diffraction data were processed with the programs DENZO (Otwinowski, 1993) and SCALPACK (Otwinowski, 1993).

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