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Spectroscopic and ITC study of the conformational change upon Ca^{2+}-binding in TnC C-lobe and TnI peptide complex from Akazara scallop striated muscle

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Abstract

Akazara scallop (*Chlamys nipponensis akazara*) troponin C (TnC) of striated adductor muscle binds only one Ca^{2+} ion at the C-terminal EF-hand motif (Site IV), but it works as the Ca^{2+}-dependent regulator in adductor muscle contraction. In addition, the scallop troponin (Tn) has been thought to regulate muscle contraction via activating mechanisms that involve the region spanning from the TnC C-lobe (C-lobe) binding site to the inhibitory region of the TnI, and no alternative binding of the TnI C-terminal region to TnC because of no similarity between second TnC-binding regions of vertebrate and the scallop TnIs. To clarify the Ca^{2+}-regulatory mechanism of muscle contraction by scallop Tn, we have analyzed the Ca^{2+}-binding properties of the complex of TnC C-lobe and TnI peptide, and their interaction using isothermal titration microcalorimetry, nuclear magnetic resonance, circular dichroism, and gel filtration chromatography. The results showed that single Ca^{2+}-binding to the Site IV leads to a structural transition not only in Site IV but also Site III through the structural network in the C-lobe of scallop TnC. We therefore assumed that the effect of Ca^{2+}-binding must lead to a change in the interaction mode between the C-lobe of TnC and the TnI peptide. The change should be the first event of the transmission of Ca^{2+} signal to TnI in Tn ternary complex.

Keywords: TnC; TnI; Ca^{2+}; NMR; ITC; CD

Contraction of vertebrate striated muscles is regulated by troponin (Tn) in a Ca^{2+}-dependent manner [1–3]. Tn contains three components: troponin C (TnC; Ca^{2+}-binding), troponin I (TnI; inhibitory through actin binding), and troponin T (TnT; tropomyosin binding) [4–6]. TnC is composed of two globular domains, N- and C-lobes, each of which consists of two EF-hand motifs [7]. Vertebrate cardiac and fast skeletal muscle TnCs bind three and four Ca^{2+} ions, respectively, per molecule [8–10], and act as the Ca^{2+}-sensor in the muscle contraction, which is associated with the binding of one or two Ca^{2+} ions in the N-terminal domain (N-lobe) of TnC. Thus, the N-lobe with one or two low-affinity Ca^{2+}-binding sites is called the regulatory domain [11]. In contrast, the C-terminal domain (C-lobe) with two high-affinity Ca^{2+}-binding sites is called the structural domain.

In invertebrate striated muscles, there are two regulatory mechanisms in muscle contraction, i.e., myosin-linked and

Abbreviations: Tn, troponin; TnC, troponin C; TnI, troponin I; TnT, troponin T; TnC N-lobe, TnC N-terminal lobe; TnC C-lobe, TnC C-terminal lobe; ITC, isothermal titration microcalorimetry; NMR, nuclear magnetic resonance; CD, circular dichroism; TCA, trichloroethanoic acid; EDTA, ethylenediaminetetraacetic acid; MOPS, 3-morpholinopropanesulfonic acid; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid).

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Tn-linked regulatory mechanisms [12–17]. Although invertebrate TnCs consist of four EF-hand motifs like their vertebrate counterparts, they can bind only one or two Ca^{2+} ions per molecule due to the lack of critical amino acids for chelation to Ca^{2+} [16]. In Akazara scallop (*Chlamys nipponensis akazara*), the TnC of striated adductor muscle binds only one Ca^{2+} ion at the C-terminal EF-hand motif (Site IV) [16], but it still works as the Ca^{2+}-dependent regulator in striated adductor muscle contraction [18,19]. TnI, which acts as the regulatory component in striated adductor muscle of Akazara scallop, also has a unique feature. The TnI consists of 292 amino acid residues, and the length of the polypeptide is greater by approximately 110 residues than that of the vertebrate skeletal muscle TnI [20]. The homologous sequence to vertebrate TnIs is only found in the C-terminal portion. The inhibitory region of TnI, which binds actin and TnC and is essential to the inhibitory function of TnI, is highly conserved. The scallop TnI has 100–133 extra residues at the N-terminus when compared with vertebrate TnIs. This extra region, whose function has not yet been characterized, has a unique sequence and contains many Glu and Arg residues. Moreover, there is no sequence similarity between the second TnC-binding region of the vertebrate fast skeletal muscle TnI and the corresponding region of scallop TnI. To shed light on this feature, functional assays have been utilized in a comparison of vertebrate TnI and Akazara scallop TnI [18]. These results suggested that scallop Tn regulates muscle contraction via an activating mechanism that involves the region spanning from the TnC C-lobe binding site to the inhibitory region of TnI, with no alternative binding of the TnI C-terminal region to TnC.

To elucidate the Ca^{2+}-dependent regulatory mechanism of striated muscle, crystal and NMR structures of TnC, both of its lobes, and the core regions of Tn ternary complexes from vertebrate striated muscle have been reported [21–28]. In contrast, there have been a limited number of structural studies of invertebrate striated muscle Tns [29–31], although the solution structure of TnC from insect flight muscle has recently been reported [32].

To clarify the molecular mechanism of the Ca^{2+}-dependent regulation of muscle contraction by scallop Tn, we have analyzed the interaction between TnC C-lobe and TnI peptide (TnIpep), which corresponds to the TnC C-lobe-binding region of vertebrate skeletal and cardiac muscle TnIs, and their Ca^{2+}-dependent conformational changes using isothermal titration microcalorimetry (ITC), nuclear magnetic resonance (NMR), circular dichroism (CD), and gel filtration chromatography.

Materials and methods

Sample preparation. Akazara scallop TnC C-terminal lobe (TnC C-lobe) was expressed and purified as described previously [29,30]. Ca^{2+}-free TnC C-lobe was prepared by TCA treatment [33]. The chemically synthesized TnIpep was purchased from Operon Biotechnologies (Tokyo, Japan). ^{15}N-labeled TnC C-lobe was obtained by the same expression system using the modified minimum medium [34] containing 1 g ^{15}N-NH_4Cl per liter culture (for ^{15}N-labeled protein) or 1 g ^{15}N-NH_4Cl and 1 g ^{13}C glucose per liter culture (for $^{13}C^{15}N$-labeled protein). Each NMR sample was prepared by mixing the concentrated 0.5 mM Ca^{2+}-free ^{15}N-labeled TnC C-lobe solution, 2.5 mM nonlabeled TnIpep solution, and 100× Ca^{2+}-stock solutions to give ratios of 0, 1, 2, 5, 6.25, 7.5, 8.25, 10, and 20 [Ca^{2+}]/[complex] in the solution of 10 mM Mops–KOH (pH 7.0), 5 mM DTT, and 100 mM KCl in 90% H_2O/10% D_2O. pH was adjusted with diluted HCl or NaOH.

NMR spectroscopy. NMR experiments were performed at 600 MHz with a Varian Inova spectrometer equipped with a *z*-axis pulse field gradient and a cold-probe. All spectra were processed using NMRPipe and drawn using NMRDraw [35]. Assignments of the NH pair in the ^{15}N–^{1}H HSQC spectrum were completed using ^{15}N–^{1}H HSQC, HNCACB, and CBCACONH spectra with Sparky (T.D. Goddard and D.G. Kneller, SPARKY 3, University of California, San Francisco).

Circular dichroism. Circular dichroism (CD) spectra were acquired on a Jasco J-720 spectropolarimeter at room temperature. All experiments were carried out using the following conditions: step resolution, 0.2 nm; speed, 50 nm min^{-1}; response time, 2 s; bandwidth, 1 nm; and number of scans, 8. The sample consisted of 20 μM TnC C-lobe complexed with TnIpep, 100 mM KCl, 0.05 mM EDTA, and 10 mM Mops–KOH (pH 7.0) for the apo state, 20 μM TnC C-lobe complexed with TnIpep, 100 mM KCl, 0.05 mM EGTA, 2 mM $MgCl_2$, and 10 mM Mops–KOH (pH 7.0) for the Mg^{2+}-bound state, and 20 μM TnC C-lobe complexed with TnIpep, 100 mM KCl, 2 mM $CaCl_2$, and 10 mM Mops–KOH (pH 7.0) for the Ca^{2+}-bound state. The background derived from the buffer was subtracted from each spectrum.

Gel filtration. Gel filtration experiments were performed on a Superdex 75 HR 10/30 (GE healthcare) column at room temperature and at a flow rate of 0.5 mL/min. The elution profile was monitored with a UV detector at 280 nm using AKTA explorer 10S (GE healthcare). The sample concentrations used were 20 μM complex in the solution containing 120 mM KCl, 0.05 mM EDTA, and 20 mM Mops–KOH (pH 7.0) for the apo form; 20 μM complex in the solution containing 120 mM KCl, 0.05 mM EGTA, 10 mM $MgCl_2$, and 20 mM Mops–KOH (pH 7.0) for the Mg^{2+}-bound form; and 20 μM complex in the solution containing 120 mM KCl, 10 mM $CaCl_2$, and 20 mM Mops–KOH (pH 7.0) for the Ca^{2+}-bound form.

Microcalorimetry. Isothermal titration microcalorimetry (ITC) was performed at 25 °C on a VP-ITC instrument (Microcal LLC, Northampton, MA, USA). TnC C-lobe was dialyzed against 150 mM KCl, 10 mM Pipes–KOH (pH 6.8), 2 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and either 0.2 mM EGTA (in the absence of Ca^{2+}) or 0.2 mM EGTA plus 0.3 mM $CaCl_2$ (in the presence of Ca^{2+}), and then clarified by centrifugation prior to use. The outer dialysate was used as a solvent for lyophilized TnIpep. The 1.4-mL sample cell was filled with 24 μM TnIpep and titrated with 260 μM TnC C-lobe. For each titration, 17 consecutive 15-mL aliquots of the C-lobe solution were injected at 220/s intervals. The heat of sample dilution was obtained from a final injection in the presence of a 2-fold molar excess of TnC C-lobe over TnIpep, and was subtracted from the C-lobe-peptide binding isotherm. Data analysis was performed using the Origin-ITC analysis package (Microcal) in "One set of sites" mode.

Results and discussion

The Ca^{2+}-dependent change of the interaction between TnC C-lobe and TnIpep was demonstrated by ITC. Fig. 1A shows a trace of the calorimetric titration of TnIpep with TnC C-lobe in the presence of Ca^{2+}. Fig. 1B shows integrated heats after subtraction of the heat of dilution and normalization with moles of TnC C-lobe injected together with single-site binding model fit. Reaction enthalpy ($\Delta H = -3.27$ kcal/mol), binding constant ($K_a = 2.93 \times 10^6\ M^{-1}$), and stoichiometry ($n = 0.907$) were