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Functional Role of Ca^{2+}-Binding Site IV of Scallop Troponin C

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Scallop troponin C (TnC) binds only one Ca^{2+}/mol and the single Ca^{2+}-binding site has been suggested to be site IV on the basis of the primary structure [K. Nishita, H. Tanaka, and T. Ojima (1994) *J. Biol. Chem.* 269, 3464–3468; T. Ojima, H. Tanaka, and K. Nishita (1994) *Arch. Biochem. Biophys.* 311, 272–276]. In the present study, the functional role of Ca^{2+}-binding site IV of akazara scallop (*Chlamys nipponensis akazara*) TnC in Ca^{2+}-regulation was investigated using a site-directed mutant with an inactivated site IV (TnC-ZEQ), N- and C-terminal half molecule mutants (TnC_N and TnC_C), and wild-type TnC (TnC_W). Equilibrium dialysis using $^{45}Ca^{2+}$ demonstrated that TnC_W and TnC_C bind 0.6–0.8 mol of Ca^{2+}/mol, but that TnC-ZEQ and TnC_N bind virtually no Ca^{2+}. The UV difference spectra of TnC_W and TnC_C showed bands at around 280–290 nm due to the perturbation of Tyr and Trp upon Ca^{2+}-binding, while TnC-ZEQ and TnC_N did not show these bands. In addition, TnC_W and TnC_C showed retardation of elution from Sephacryl S-200 upon the addition of 1 mM $CaCl_2$, unlike TnC-ZEQ and TnC_N. These results indicate that Ca^{2+} binds only to site IV and that Ca^{2+}-binding causes structural changes in both the whole TnC molecule and the C-terminal half molecule. In addition, TnC_W, TnC-ZEQ, and TnC_C, but not TnC_N, were shown to form soluble complexes with scallop TnI at physiological ionic strength. On the other hand, the Mg-ATPase activity of reconstituted rabbit actomyosin in the presence of scallop tropomyosin was inhibited by scallop TnI and recovered by the addition of an equimolar amount of TnC_W, TnC-ZEQ, or TnC_C, but not TnC_N. These results imply that the site responsible for the association with TnI is located in the C-terminal half domain of TnC. Ternary complex constructed from scallop TnT, TnI, and TnC_W conferred Ca^{2+}-sensitivity to the Mg-ATPase of rabbit actomyosin to the same extent as native troponin, but the TnC_N-TnT-TnI and TnC-ZEQ-TnT-TnI complexes conferred no Ca^{2+}-sensitivity, while the TnC_C-TnT-TnI complex conferred weak Ca^{2+}-sensitivity. Thus, the major functions of scallop TnC, such as Ca^{2+}-binding and interaction with TnI, are located in the C-terminal domain, however, the full Ca^{2+}-regulatory function requires the presence of the N-terminal domain.

Key words: scallop troponin C, Ca^{2+}-regulatory function, recombinants, Mg-ATPase activity.

Muscle contraction is regulated by the association and dissociation of intracellular Ca^{2+} with Ca^{2+}-regulatory proteins that are specific to the animal species and muscle type. In vertebrate striated muscles, the Ca^{2+}-responsive regulatory protein is troponin, which consists of three distinct subunits, *i.e.*, a tropomyosin-binding subunit (TnT), an inhibitory subunit (TnI), and a Ca^{2+}-binding subunit (TnC). Troponin locates on thin filaments together with tropomyosin and regulates actin–myosin interaction through Ca^{2+}-binding to TnC (*1–6*). On the other hand, some invertebrate muscles, particularly molluscan muscles, are known to be regulated by the myosin-linked regulatory system mediated through Ca^{2+}-binding to myosin light chains (*7–11*). Scallop muscle has been demonstrated to possess both myosin-linked and actin-linked systems (*12–16*). Recently, the physiological significance of the coexistence of the two systems in scallop adductor muscle was investigated using CDTA-treated scallop myofibrils (*17*). Scallop troponin was shown to confer Ca^{2+}-sensitivity to the myofibrillar Mg-ATPase by increasing the activity at higher Ca^{2+} concentrations, while the regulatory light chain conferred Ca^{2+}-sensitivity mainly by inhibiting the activity at lower Ca^{2+} concentrations (*17*). In addition, troponin was shown to exhibit its function only in the temperature range of 5–15°C, which is close to the temperature range of the scallop's natural habitat.

On the other hand, scallop troponin subunits have been reported to possess some distinct properties from their vertebrate counterparts, *e.g.*, striated adductor muscle TnI has an N-terminus that is approximately 130 residues longer than that of vertebrate TnI (*18*), while the N-terminus of TnT is 16–22 residues shorter and the C-terminus is 78–79 residues longer than those of vertebrate TnTs (*19*). Further, scallop TnC binds only one Ca^{2+}/mol and the single Ca^{2+}-binding site is believed to be site IV, which corresponds to

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Abbreviations: TnI, troponin I; TnT, troponin T; TnC, troponin C; CDTA, *trans*-1,2-cyclohexanediamine-*N,N,N′,N′*-tetraacetic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid.

one of the so-called "structural sites" in vertebrate TnC (*20*, *21*). These facts suggest that the molecular mechanism of Ca^{2+}-regulation of scallop troponin is somehow different from that of vertebrate troponin. In fact, vertebrate skeletal troponin requires the binding of Ca^{2+} to N-terminal sites I and II on TnC to trigger contraction (*22*, *23*); however, this mechanism is not applicable to the scallop troponin system since the N-terminal domain of scallop TnC seems to be incapable of binding Ca^{2+}.

In the present study, we constructed a mutant TnC with an inactivated site IV and N- and C-terminal half molecule mutants. Using these, we have confirmed that site IV is the single Ca^{2+}-binding site on akazara scallop TnC. We also investigated the roles of the N- and C-terminal domains in the Ca^{2+}-regulatory function and their biochemical properties.

MATERIALS AND METHODS

Mutagenesis was performed with the akazara scallop TnC cDNA, "AC2K" (*21*, accession number in DDBJ/GenBank/EMBL: D85883), and an Amersham Sculptor *in vitro* mutagenesis kit. A bacterial expression system consisting of a pET-16b plasmid and *Escherichia coli* BL21 (DE3) was purchased from Novagen. Restriction endonucleases, T4 ligase, and other modifying enzymes were purchased from TaKaRa. The nucleotide sequence of the cDNA was determined using a PE Biosystems 310 Genetic Analyzer. The amino acid sequences of the recombinant TnCs were determined with a PE Biosystems 473A Protein Sequencer. Troponin, troponin subunits, and tropomyosin of akazara scallop striated adductor muscle were prepared as reported previously (*15*, *24*). Reconstituted troponins were constructed by combining equimolar amounts of akazara scallop TnT, TnI, and recombinant TnCs, followed by successive dialyses against, first, 6 M urea, 0.5 M KCl, 10 mM Tris-HCl (pH 7.6), and 5 mM 2-mercaptoethanol for 6 h, then 0.5 M KCl, 10 mM Tris-HCl (pH 7.6), and 5 mM 2-mercaptoethanol for 6 h, and finally 0.05 M KCl, 10 mM Tris-HCl (pH 7.6), and 5 mM 2-mercaptoethanol for 12 h. Rabbit myosin and actin were prepared by the methods of Perry (*25*) and Spudich and Watt (*26*), respectively. The amount of Ca^{2+}-binding to TnC was determined at 20°C by the equilibrium dialysis method using micro-dialysis cells and $^{45}CaCl_2$ as reported previously (*15*). The UV-absorption and difference UV-absorption spectra for TnC were measured at 20°C as described previously (*15*). The free Ca^{2+} concentration was varied with Ca-EGTA buffer adopting the apparent binding constant of 8.45×10^5 M^{-1} (*27*). Gel-filtration of TnC in the presence or absence of $CaCl_2$ was carried out at 4°C on a column of Sephacryl S-200 (1.8 × 110 cm) pre-equilibrated with 50 mM KCl, 10 mM Tris-HCl (pH 7.6), 2 mM 2-mercaptoethanol, and 1 mM $CaCl_2$ or 0.5 mM EGTA. The solubilization of scallop TnI by TnC at low ionic strength was examined as previously reported (*28*). SDS–polyacrylamide gel electrophoresis was carried out by the method of Porzio and Pearson (*29*). The Mg-ATPase activity was measured in a solution containing 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 20 mM Tris maleate (pH 6.8), 0.2 mM EGTA, and various concentrations of $CaCl_2$, 0.1 mg/ml rabbit myosin, 0.05 mg/ml rabbit F-actin, 0.025 mg/ml akazara scallop tropomyosin, and 0.05 mg/ml reconstituted troponins. Inorganic phosphate liberated by ATP hydrolysis was determined by the method of Youngburg and Youngburg (*30*). Protein concentration was determined by the biuret method (*31*) using bovine serum albumin fraction V as a standard protein.

RESULTS

Construction of Akazara Scallop TnC Mutants—A cDNA clone fully encoding the akazara scallop TnC protein, "AC2K" (*21*), was used as a template for mutagenesis. AC2K consists of 1,987 bp including an open reading frame of 462 bp at nucleotide positions 12–473, which encodes 152 amino acids in addition to initiation and termination codons. All mutagenesis was achieved with the single strand AC2K cDNA rescued from recombinant pBluescript II KS (–) and specific oligonucleotide primers. In order to inactivate Ca^{2+}-binding site IV of scallop TnC, Glu_{142} at the -Z coordinating position of the Ca^{2+}-binding loop was replaced with Gln by changing the Glu codon (GAA) to a Gln codon (CAG) with a 5′-TGTAGATTATGAACAGTTCAAAT-3′ primer (nucleotides for mutagenesis are underlined),

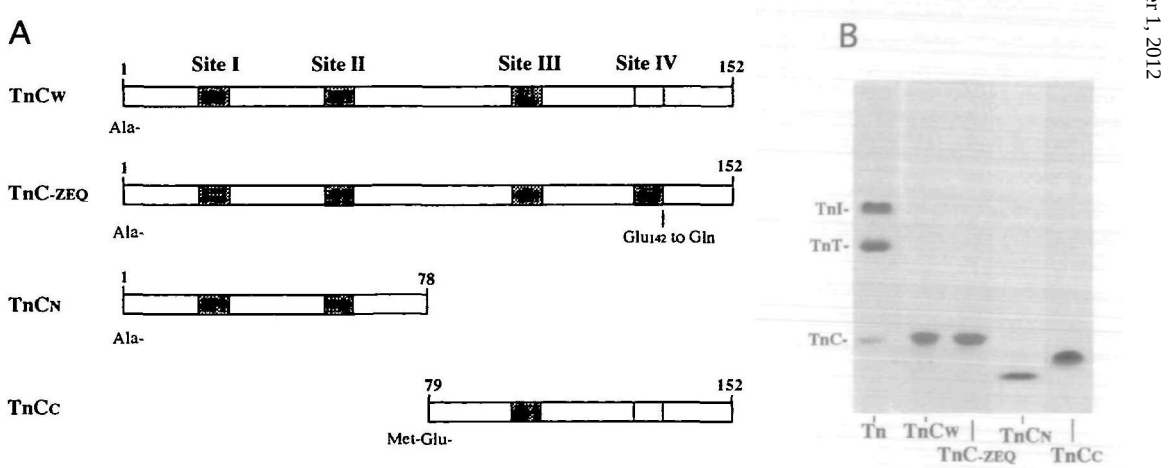

Fig. 1. **Schematic representation of TnC mutants and SDS-PAGE for TnC mutants.** A, amino acids changed by DNA manipulations are indicated. Shaded regions show the inactivated Ca^{2+}-binding sites. Amino acid residue numbers corresponding to those of native TnC are shown above the figure. B, SDS-PAGE of TnC mutants.

thus obtaining the cDNA for TnC-ZEQ (see Fig. 1). To generate the N-half molecular mutant, TnC_N, which corresponds to amino acid positions 1–78, a termination codon "TGA" followed by a *Bam*HI recognition sequence "GGATCC," was introduced into the central helix region of the cDNA with a 5′-GAATGGAAGCTTTGAGAGGATCCGGACGAAAGG-3′ primer. Thus, the codon for Lys_{79} was changed to a termination codon. The C-terminal half molecular mutant, TnC_C, which corresponds to amino acid positions 79–152, was generated by introducing an *Nco*I site, whose inner "ATG" sequence is available as a translational initiation codon in the pET-16b plasmid, at the position of Lys_{79} in the central helix region of the cDNA with a 5′-GAATGGAAGCCCATGGAGGATTTG-3′ primer. In addition to these mutations, the 5′-terminus of the translated regions of TnC_N and wild type TnC (TnC_W) cDNAs were altered to *Nco*I sites with a 5′-GCCTTCCACCATGGCTGAC-3′ primer. A *Bam*HI site was introduced into the 3′-untranslated regions of the TnC-ZEQ, TnC_C, and TnC_W cDNAs with a 5′-GGGGTACGGATCCGGTTCT-3′ primer. These mutated cDNAs were digested with *Nco*I and *Bam*HI and ligated into the cloning site between the *Nco*I and *Bam*HI sites of pET-16b. These manipulations to the cDNAs were expected to result in some replacements of the N-terminal amino acids in the expressed proteins, *i.e.*, the N-terminal met-Ser of native TnC is replaced by met-Ala in TnC_W, TnC-ZEQ, and TnC_N, and the internal Lys_{79}-Glu_{80} sequence in native TnC by the N-terminal met-Glu_{80} in TnC_C ("met" means the initiation Met). The recombinant TnC proteins were expressed in *E. coli* BL21 (DE3) in a 1,000 ml overnight culture by induction with 1 mM isopropyl-β-D(−)-thiogalactopyranoside at 37°C for 2 h as described previously (*32*). The bacteria were harvested by centrifugation at 5,000 ×*g* for 15 min, and suspended in 50 ml of 8% sucrose, 10 mM Tris-HCl (pH 8.0), 50 mM EDTA, 0.5% Triton X-100, and 2 mM phenylmethylsulfonyl fluoride. The recombinant TnCs were extracted from the bacteria by repeated freeze and thaw, and the TnCs obtained in the soluble fraction were purified by subsequent column chromatographies on DEAE-Toyopearl 650 M and Sephacryl S-200 (*24*, *32*). The yields of TnC_W, TnC-ZEQ, and TnC_N were usually 50–70 mg from 1,000 ml culture, while that of TnC_C was 5–10 mg. The SDS-gel electrophoretic patterns of the purified TnCs are shown in the inset of Fig. 1. It is noteworthy that TnC_N showed greater mobility than TnC_C although their molecular weights do not differ by very much (approx. 8,993 and 8,517 for TnC_N and TnC_C, respectively). Since the electrophoretic mobility of proteins in SDS-gels is generally known to be affected by differences in the amounts of charged amino acids, the difference in mobility between TnC_N and TnC_C on SDS-PAGE may be attributable to the large difference in contents of acidic and basic amino acids between the two proteins, *i.e.*, TnC_N contains 9 Asp, 8 Glu, 12 Lys, and 2 Arg, while TnC_C contains 10 Asp, 12 Glu, 7 Lys, and 3 Arg.

Determination of the N-terminal 25 amino acid sequences of the recombinant TnCs using a protein sequencer confirmed that the cDNAs were correctly translated as expected above. The N-terminal Met was cleaved off in TnC_W, TnC-ZEQ, and TnC_N but remained in TnC_C. Up to now, we have not detected any differences in properties between native TnC and TnC_W.

Ca^{2+}-Binding of TnC Mutants—The amount of Ca^{2+} bound to akazara scallop TnC at 10^{-4} M Ca^{2+} was estimated to be 0.7 mol/mol, which corresponds physiologically to 1 mol/mol, and the single Ca^{2+}-binding site was suggested to be site IV from the sequence analysis (*15*, *20*, *21*).

TABLE I. **Calcium binding of akazara scallop recombinant TnCs.** The number of Ca^{2+} bound to the akazara scallop recombinant TnCs was measured by the equilibrium dialysis method in a medium containing 0.1 M KCl, 20 mM MOPS-KOH (pH 6.8), and 1 mg/ml of TnC_W, TnC-ZEQ, TnC_N, or TnC_C. EGTA, in the presence of 1 mM EGTA; Ca^{2+}, in the presence of 0.1 mM $CaCl_2$; Ca^{2+} + Mg^{2+}, in the presence of 0.1 mM $CaCl_2$ and 2 mM $MgCl_2$. The average values of four experiments are shown with the mean deviation.

	Number of Ca^{2+} bound (mol/mol ± SD)		
	EGTA	Ca^{2+}	Ca^{2+} + Mg^{2+}
TnC_W	0.04 ± 0.05	0.75 ± 0.06	0.68 ± 0.02
TnC-ZEQ	0.06 ± 0.02	0.05 ± 0.01	0.02 ± 0.01
TnC_N	0.02 ± 0.01	0.04 ± 0.01	0.02 ± 0.01
TnC_C	0.04 ± 0.02	0.67 ± 0.02	0.63 ± 0.02

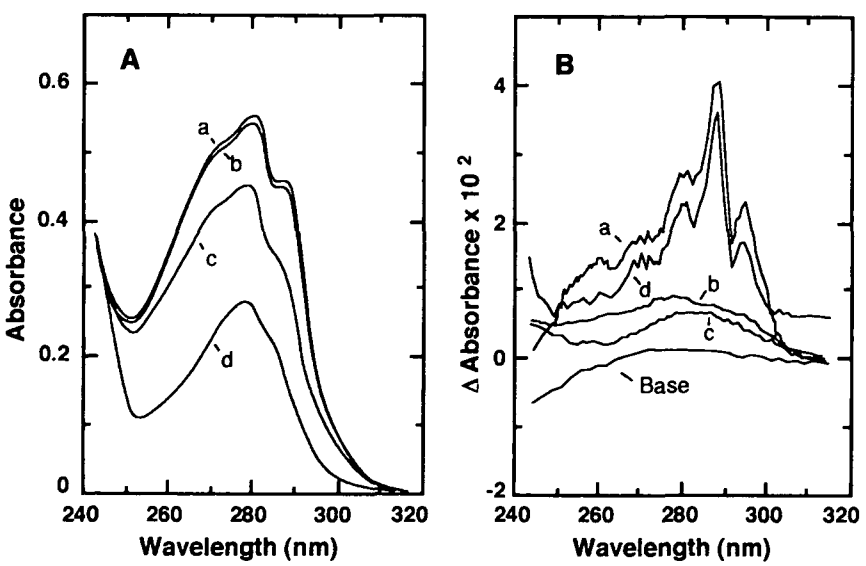

Fig. 2. **UV-absorption and difference UV-absorption spectra of TnC mutants.** Akazara scallop recombinant TnCs (0.5 mg/ml) were dialyzed against 50 mM KCl, 20 mM MOPS-KOH (pH 6.8), and 0.1 mM EGTA. A, the UV-absorption spectra of TnC_W (a), TnC-ZEQ (b), TnC_N (c), and TnC_C (d). B, Ca^{2+}-induced difference spectra of TnC_W (a), TnC-ZEQ (b), TnC_N (c), and TnC_C (d). The sample and reference cells contained 0.1 mM EGTA and 0.1 mM EGTA plus 0.3 mM $CaCl_2$, respectively.

Therefore, the amount of Ca^{2+} bound to TnC_W, TnC-ZEQ, TnC_N, and TnC_C was determined by the equilibrium dialysis method. As shown in Table I, TnC_W and TnC_C bind 0.6–0.8 mol of Ca^{2+}/mol, however, TnC-ZEQ and TnC_N bind essentially no Ca^{2+}. These results coincide with the previous expectation (*20, 21*) that the single Ca^{2+}-binding site of scallop TnC is site IV in the C-terminal domain. The Ca^{2+}-binding of TnC_W and TnC_C was hardly influenced by the presence of 2 mM $MgCl_2$, indicating that the Ca^{2+}-binding to site IV is Ca^{2+}-specific as previously reported for native TnC (*15, 20*).

Difference UV-Absorption Spectra of TnC Mutants—It has been shown that akazara scallop TnC exhibits a Ca^{2+}-induced UV-difference spectrum due to the perturbation of Tyr and Trp at 10^{-5}–10^{-4} M Ca^{2+} (*15, 33*). Therefore, the UV-absorption and difference UV-absorption spectra of the TnC mutants were determined. As shown in Fig. 2A, the UV-absorption spectra of the TnC mutants showed different profiles. This may be due to differences in the content and molecular extinction coefficients of the aromatic amino acids among the mutants, *i.e.*, TnC_W and TnC-ZEQ contain 7 Phe, 4 Trp, and 1 Tyr; TnC_N contains 5 Phe, 3 Trp, and no Tyr; TnC_C contains 2 Phe, 1 Trp, and 1 Tyr. Figure 2B shows that difference UV-absorption spectra of TnC_W and TnC_C are caused by the addition of 2×10^{-4} M Ca^{2+}. On the other hand, TnC-ZEQ and TnC_N show practically no difference UV-absorption. These results are consistent with the results of Ca^{2+}-binding measurement on TnC mutants (Table I). It is noteworthy that both the profile and extent of the difference absorption spectra between TnC_W and TnC_C were similar. Since TnC_C contains only 1 Trp (Trp_{109} in native TnC) in the region corresponding to the F-helix of vertebrate TnC (*21*), the difference spectrum observed in either TnC_C or TnC_W may reflect conformational changes around the putative F-helix region of the scallop TnC. Since the Ca^{2+}-binding site III flanked by E- and F-helices binds no Ca^{2+}, Ca^{2+}-binding to site IV is considered to cause conformational changes in the entire C-terminal domain involving both sites III and IV. Then, the relative extent of the difference spectrum between the two peak signals at 289 and 292 nm of TnC_W and TnC_C was plotted against free Ca^{2+} concentration (Fig. 3). Judging from the Ca^{2+} concentrations needed to produce half maximal changes in the difference absorbance spectra, TnC_C appears to have a slightly lower affinity for Ca^{2+} (Ca^{2+} concentration for half maximal change, approx. 50 μM) than TnC_W (approx. 25 μM). These results indicate that the absence of the N-terminal domain does not greatly lower the affinity of site IV for Ca^{2+}.

Effects of Ca^{2+} on Elution Volume of TnC Mutants on Gel-Filtration—Previously, we showed that Ca^{2+}-induced structural changes in akazara scallop TnC are detectable as a retardation of elution on gel-filtration (*33*). Therefore, the effects of Ca^{2+} on the elution volume of the TnC mutants on Sephacryl S-200 gel-filtration were investigated. As shown

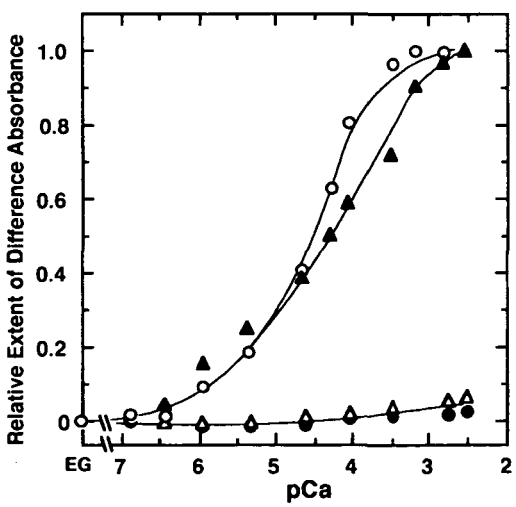

Fig. 3. **Ca^{2+}-dependent changes in difference UV-absorption spectra of TnC mutants.** The difference spectra of TnC_W (○), TnC-ZEQ (●), TnC_N (△), and TnC_C (▲) were measured at 20°C in the presence of various concentrations of Ca^{2+}. The relative extent of the difference absorption was estimated from the difference between the positive peak signal at 289 nm and the negative peak signal at 292 nm.

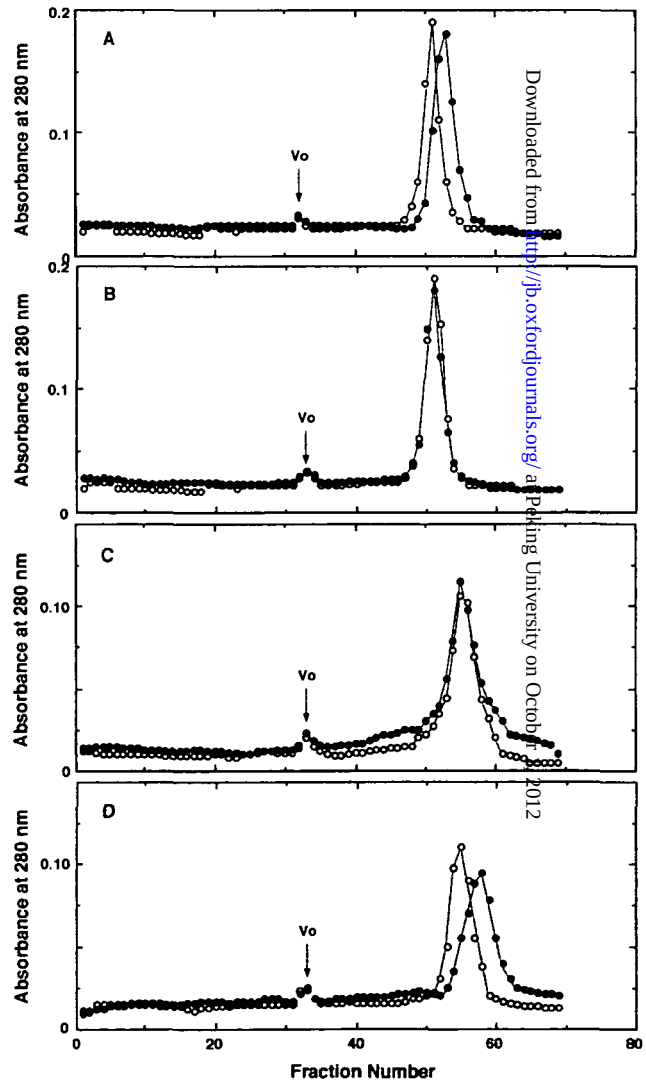

Fig. 4. **Gel-filtration of TnC mutants on Sephacryl S-200.** Each of TnC mutants (3 mg) dissolved in 1.0 ml of 50 mM KCl, 10 mM Tris-HCl (pH 7.5), and 2 mM 2-mercaptoethanol, and 0.5 mM EGTA (○) or 1 mM $CaCl_2$ (●) was applied to Sephacryl S-200 column (1.8 × 110 cm) and eluted with the same buffer. A, TnC_W; B, TnC-ZEQ; C, TnC_N; D, TnC_C.

in Fig. 4, the elution peak of TnC_W was at fraction 51 in the absence of Ca^{2+}, and this shifted to fraction 53 in the presence of Ca^{2+}, indicating that the structural changes make the molecule more compact. In the case of TnC_C, a similar result was obtained, *i.e.*, the elution peak of TnC_C at fraction 55 in the absence of Ca^{2+} shifted to fraction 58 by the presence of Ca^{2+}. On the other hand, the elution peaks of TnC-ZEQ and TnC_N at fractions 51 and 55, respectively, remained unchanged regardless of the presence or absence of Ca^{2+}. These results indicate that Ca^{2+}-binding to site IV causes conformational changes in the C-terminal domain of TnC as well as in intact TnC (*33*).

Solubilization of TnI by TnC Mutants—Akazara scallop TnI is insoluble at physiological ionic strength, but becomes soluble if it associates with TnC (*28*). Thus, TnC_W, TnC-ZEQ, TnC_N, and TnC_C were added in various molar ratios to the akazara scallop TnI in a medium of 0.6 M KCl, 10 mM Tris-HCl (pH 7.6), and the mixtures were diluted with 9 volumes of cold distilled water. Then, the turbidity of the mixtures was determined at 15°C as absorbance at 330 nm. As shown in Fig. 5, TnI was completely solubilized by the addition of an equimolar amount of TnC_W, TnC-ZEQ, or TnC_C; however, it was not solubilized by TnC_N. This indicates that the TnI-binding site(s) of scallop TnC are located mainly in the C-terminal domain.

Restoration of TnI-Inhibition of ATPase by TnC Mutants—The inhibition of the Mg-ATPase activity of actomyosin-tropomyosin by TnI is neutralized by the association of TnI with TnC (*28, 34, 35*). Therefore, the effects of TnC mutants on the inhibition of scallop TnI were investigated. As shown in Fig. 6, the Mg-ATPase activity of rabbit actomyosin-scallop tropomyosin, which had been repressed by scallop TnI, was recovered by the addition of TnC_W, TnC-ZEQ, or TnC_C, but not TnC_N, regardless of the presence or absence of Ca^{2+}. Further, the degree of recovery was comparable and reached a maximum at a molar ratio of approx. 1:1, suggesting that the neutralization of the Mg-ATPase activity is due to the formation of equimolar complexes between TnI and the C-terminal domain of TnC. Since TnC-ZEQ was capable of restoring TnI inhibition and the restoration by TnC_W and TnC_C required no Ca^{2+}, the occupation of site IV by Ca^{2+} seems unnecessary for the neutralization of TnI.

Ca^{2+}-Regulation Ability of Ternary Complexes—The Ca^{2+}-regulation ability of ternary complexes constructed from scallop TnT, TnI, and TnC mutants on rabbit actomyosin-scallop tropomyosin Mg-ATPase was investigated. Complexes constructed as described in "MATERIALS AND METHODS" were added to rabbit skeletal actomyosin together with akazara scallop tropomyosin, and the Mg-ATPase activity of the actomyosin was measured as a function of Ca^{2+} concentration. As a result, the complex containing TnC_W

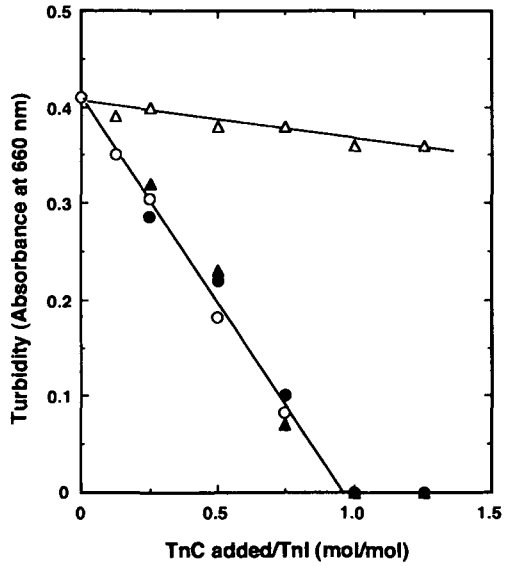

Fig. 5. **Solubilization of scallop TnI with TnC mutants.** TnC_W (○), TnC-ZEQ (●), TnC_N (△), and TnC_C (▲) were added to akazara scallop TnI in the molar ratios indicated on the abscissa in a medium of 0.6 M KCl and 10 mM Tris-HCl (pH 7.6); the mixture was diluted to 0.06 M KCl with cold distilled water, and then the absorbance at 330 nm was measured.

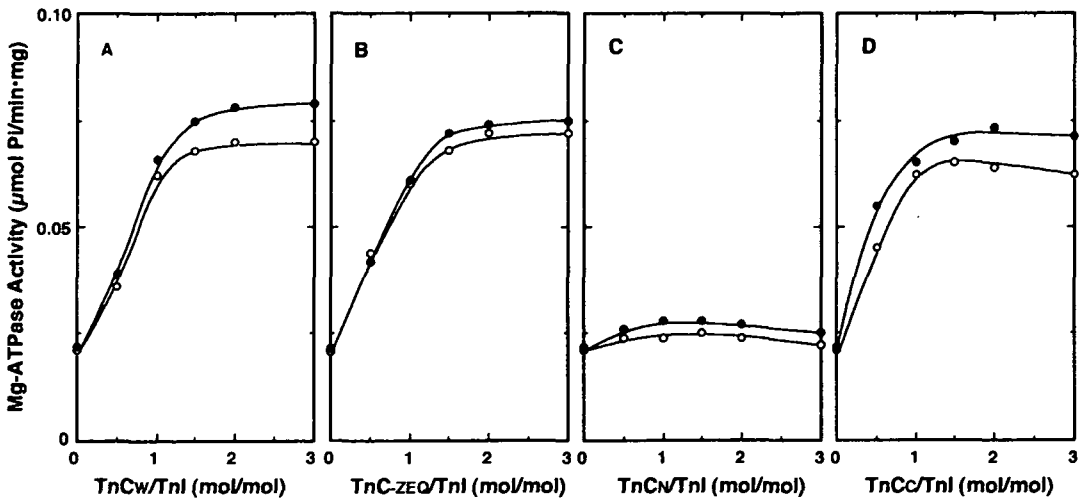

Fig. 6. **Effects of TnC mutants on actomyosin Mg-ATPase activity inhibited by TnI.** Mg-ATPase activity was measured at 15°C in a mixture containing 0.05 M KCl, 20 mM Tris maleate (pH 6.8), 2 mM $MgCl_2$, 0.2 mM EGTA (○) or 0.2 mM EGTA plus 0.3 mM $CaCl_2$ (●), 0.1 mg/ml rabbit myosin, 0.05 mg/ml rabbit F-actin, 0.025 mg/ml akazara scallop tropomyosin, 0.028 mg/ml akazara scallop TnI, and various concentrations of TnC_W (A), TnC-ZEQ (B), TnC_N (C), and TnC_C (D).

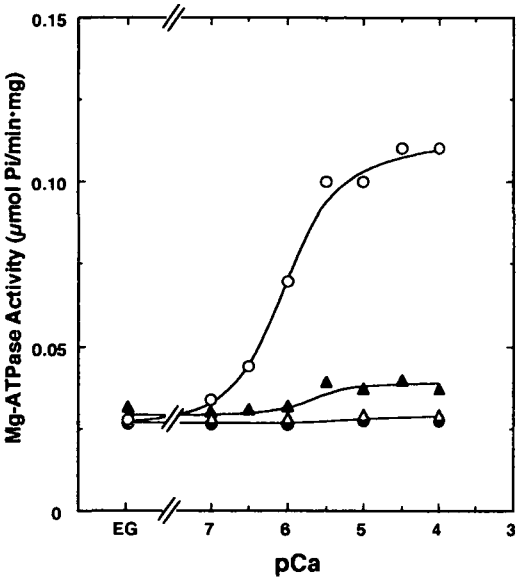

Fig. 7. **Ca^{2+}-regulation ability of ternary complexes constructed from scallop TnT-TnI and TnC mutants.** Ternary complexes were constructed from akazara scallop TnT, TnI, and TnC_W (○), TnC-ZEQ (●), TnC_N (△), or TnC_C (▲) in an equimolar ratio as described in "MATERIALS AND METHODS." The complexes were added to rabbit reconstituted actomyosin together with akazara scallop tropomyosin. Then, the Mg-ATPase activity was measured at 15°C in a solution containing 50 mM KCl, 20 mM Tris maleate (pH 6.8), 2 mM $MgCl_2$, 0.1 mM EGTA with various concentrations of $CaCl_2$, 0.2 mg/ml rabbit myosin, 0.1 mg/ml rabbit F-actin, 0.05 mg/ml akazara scallop tropomyosin, and 0.05 mg/ml each of ternary complexes. EG, in the presence of 0.2 mM EGTA. The free Ca^{2+} concentration in the reaction mixture was determined using an apparent binding constant of 8.45×10^5 M^{-1} for the Ca-EGTA complex (*27*).

(TnT-TnI-TnC_W) conferred Ca^{2+}-sensitivity on the Mg-ATPase activity to the same extent as native scallop troponin showing half-maximal activation at around 1 μM Ca^{2+} (Fig. 7). On the other hand, the complex containing TnC-ZEQ or TnC_N conferred no Ca^{2+}-sensitivity on the actomyosin Mg-ATPase activity. The complex of TnT-TnI-TnC_C conferred slight Ca^{2+}-sensitivity by activating the Mg-ATPase activity at 10^{-5}–10^{-4} M Ca^{2+}. These results indicate that the full Ca^{2+}-regulatory function of scallop TnC requires the presence of both the N- and C-terminal domains even though the N-terminal domain does not bind Ca^{2+}.

DISCUSSION

In the present study, we confirmed that the single Ca^{2+}-binding site of akazara scallop TnC is site IV using TnC mutants, *i.e.*, TnC-ZEQ with an inactivated site IV, and the N- and C-terminal half mutants, TnC_N and TnC_C. It has been reported that the number of active Ca^{2+} binding sites on TnC varies depending on the TnC species. For example, vertebrate fast muscle TnC possesses four active Ca^{2+}-binding sites. Of them, N-terminal sites I and II are known as the regulatory sites, while C-terminal sites III and IV are regarded as structural sites (*5*, *6*, *36–38*). On the other hand, vertebrate cardiac muscle TnC possesses an inactivated site I due to the replacement of some amino acids essential for the coordination of divalent cations; thus this TnC can bind three Ca^{2+} ions (*39*, *40*). Further, arthropod TnCs possess two active Ca^{2+}-binding sites at sites II and IV, and site II is suggested to be the regulatory site (*41–44*). Therefore, scallop TnC is the only TnC possessing a single active Ca^{2+}-binding site at site IV among the TnCs reported so far. Based on the Ca^{2+}-sensitizing ability of TnC mutants on the rabbit reconstituted actomyosin Mg-ATPase activity, site IV can be concluded to be the regulatory site. In addition, it may also play structural roles since treatment of scallop myofibrils with a strong divalent cation chelator, CDTA, causes the dissociation of TnC from the thin filament (*17*). On the other hand, Ca^{2+} is not required for the binding of TnC to TnI (Figs. 5 and 6), and site IV appears to be a Ca^{2+}-specific site (Table I), indicating that the structural role of site IV remains obscure but different from the case of vertebrate TnCs.

Recently, models for the Ca^{2+}-dependent interaction between vertebrate skeletal TnC and TnI have been proposed (*22*, *23*). According to these models, Ca^{2+}-binding to the N-terminal region of TnC is a key reaction causing the conformational changes in TnC and subsequent events for the interaction of TnI and TnC and regulatory actions. Therefore, these models are not applicable to the scallop TnC-TnI system since the Ca^{2+}-binding ability of the N-terminal domain is lost in scallop TnC. Actually, the ternary complex constructed from akazara scallop TnC and rabbit skeletal TnT and TnI does not confer Ca^{2+}-sensitivity to the rabbit actomyosin Mg-ATPase activity (*33*). In addition, the primary structure around the inhibitory region of the akazara scallop TnI, especially the C-terminal portion of the inhibitory segment (corresponding to residues 117–127 of rabbit TnI), shows low homology to rabbit TnI (*18*). Taking these facts into consideration, we conclude that the regulatory mechanism of scallop troponin differs from that of vertebrate troponin. In this view, the coexistence of troponin and the myosin-linked regulatory system may have allowed broad variation in the properties and functions of scallop troponin during evolution.

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