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Ca²⁺-binding properties and regulatory roles of lobster troponin C sites II and IV

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Abstract:

Invertebrate troponin C typically contains Ca²⁺-specific binding sites, sites II and IV, in the N- and C-terminal domains, respectively. To investigate the roles of these sites for Ca²⁺-dependent regulation of muscle contraction, we generated lobster troponin C mutants, and analyzed their Ca²⁺-binding properties and regulatory effects on actomyosin-tropomyosin Mg-ATPase activity. The results suggest that Ca²⁺ binding to site IV is responsible for regulation at relatively low Ca²⁺ concentrations, while site II has an essential role in full activation at higher Ca²⁺ concentrations.

Keywords:

Troponin; Muscle; Ca²⁺-regulation; Invertebrate; Arthropod; **Isoform**

Highlights:

- >Ca²⁺-binding site II of lobster troponin C binds Ca²⁺ and also binds Mg²⁺ very weakly.
- >Site IV specifically binds Ca²⁺ with higher affinity than site II.
- >Ca²⁺ binding to site II as well as site IV is important in regulating muscle contraction.
- >Regulatory site selection is Ca²⁺ concentration-dependent.

1. Introduction

Vertebrate striated muscle contraction is regulated by a thin filament linked system, troponin (Tn)-tropomyosin (Tm) [1]. Tn is composed of three subunits: troponin C (TnC) binds intracellular Ca^{2+} ; troponin I (TnI) inhibits contraction; and troponin T (TnT) binds the Tn complex to Tm [2]. The N- and C-terminal domains of TnC each contain two EF-hand Ca^{2+} -binding sites. Ca^{2+} -binding sites III and IV in the C-terminal domain strongly bind Ca^{2+} with an association constant (K_{Ca}) of $\sim 10^7 \text{ M}^{-1}$, and also bind Mg^{2+} with K_{Mg} of $\sim 10^3$ to 10^4 M^{-1} [3]. These sites are likely to be occupied by Mg^{2+} under physiological conditions. On the other hand, N-terminal sites I and II specifically bind Ca^{2+} with a K_{Ca} of $\sim 10^5 \text{ M}^{-1}$ [3], and Ca^{2+} -binding to these sites leads to exposure of a hydrophobic pocket [4] that attracts an amphiphilic helix in the C-terminal portion of TnI. This movement releases TnI, allowing actin to interact with myosin [5].

The Tn-Tm complex has been found in many invertebrates, such as protochordates, arthropods, nematodes, and molluscs [6]. However, invertebrate TnCs have lost Ca^{2+} -binding in one to three Ca^{2+} -binding sites as a result of amino acid substitutions in EF-hand motif Ca^{2+} -binding loops. The major isoforms of TnC from crustacean [7-10] and nematode [11,12] bind two Ca^{2+} ions at sites II and IV. These sites were experimentally shown or predicted from their amino acid sequences to bind Ca^{2+} but not Mg^{2+} , and it has been suggested that muscle contraction should be triggered by Ca^{2+} binding to site II as in the case of vertebrate Tn. However, little is known about the physiological effects of Ca^{2+} binding to site IV in these animals. Moreover, forms of TnC that bind only one Ca^{2+} at site IV have been reported. For instance, TnC-F1 found in insect flight muscle can bind a single Ca^{2+} at site IV [13,14]. Similarly, molluscan TnC binds one Ca^{2+} at site IV and yet scallop Tn activates the actomyosin-Tm Mg-ATPase as effectively as vertebrate Tn in a Ca^{2+} -dependent manner [15-17]. These observations suggest that there may be a novel molecular regulatory mechanism for muscle contraction involving Ca^{2+} -binding to site IV of TnC in some invertebrates.

Here, we report the Ca^{2+} -binding properties of sites II and IV of lobster TnC isoforms, and provide evidence for a regulatory role of Ca^{2+} binding at both of these sites.

2. Materials and methods

2.1. Preparation of muscular proteins

Lobster Tn and Tm were prepared from the deep abdominal flexor muscles by the method of Nishita and Ojima [18] and the isoforms of Tn components were separated by the method of Miegel et al. [19]. This muscle contains three major isoforms of TnC (TnC-1, TnC-2a and TnC-2b), five isoforms of TnI (TnI-1 to TnI-5), and a single isoform of TnT [19]. However, there have been no reports demonstrating the association of these isoforms into a Tn complex. In this study, we used TnI-4 since it is the most abundant isoform [19]. Rabbit fast skeletal myosin and actin were

prepared as described previously [17].

2.2. cDNA cloning and construction of plasmids expressing TnC mutants

The amino acid sequences of TnC-2a and 2b are more similar to each other than either sequence is to TnC-1 (Fig. 1A; see also Ref. 9). Therefore, we chose TnC-1 and TnC-2a as templates for generating mutants with predicted functional differences.

Total RNA was extracted from the muscle using RNAiso plus (Takara Bio, Ohtsu, Japan) and used for cDNA synthesis with the TaKaRa RNA PCR kit (AMV) Ver. 3.0 (Takara Bio). Based on the protein sequences [9], we designed degenerate sense primers, TnC1Fw (5'-ATGGAYACNYTNGAYGARGAYCARGTNCARGC-3') and TnC2aFw (5'-ATGGAYWSNYTNGAYGARGARCARATHGGNGC-3'), and antisense primers, TnC1Rv (5'-NCCRTTCATCATYTTTCATRAAYTCRTTRAARTC-3') and TnC2aRv (5'-NCCRTTCATCATYTTCCATRAAYTCRTTRAARTC-3') to amplify the cDNA encoding TnC-1 and TnC-2a, respectively. The PCR products were sequenced and then subcloned into *NcoI*-*Bam*HI sites of pET-16b (Merck, Darmstadt, Germany) to generate plasmids expressing TnC-1 (WT) and TnC-2a (WT) without any additional tag-sequence. We also prepared plasmids expressing mutants, TnC-1 (-IV) and TnC-2a (-IV), which bind Ca²⁺ at site II but not at site IV as a result of the replacement of -Z-coordinating Glu₁₄₃ with Gln, as well as mutants TnC-1 (-II) and TnC-2a (-II), which bind Ca²⁺ at site IV but not at site II as a result of Gln substitutions at Glu₆₇ (Fig. 1B). The mutations were introduced into expression plasmids for TnC-1 (WT) and TnC-2a (WT) using PrimeSTAR mutagenesis basal kit (Takara Bio).

2.3. Expression and purification of recombinant proteins

Escherichia coli BL21 Rosetta2 (DE3) cells (Merck) were transformed with the expression plasmids. Induction of expression and extraction of recombinant proteins were performed as described previously [17]. TnC was purified from the crude extracts by Toyopearl DEAE-650M (Tosoh, Tokyo, Japan) column chromatography and subsequent ammonium sulfate fractionation at 75 to 90% saturation. Although the mobilities of the mutant proteins on SDS-PAGE (Fig. 1C) varied somewhat, the molecular masses determined by MALDI-TOF mass spectrometry were in agreement with those calculated from the sequences within an error of less than 0.15%.

2.4. Measurement of Mg-ATPase activity

Reconstituted Tn composed of recombinant TnC, native lobster TnT, and TnI was prepared as described previously [17] and was subjected to gel filtration with a Superdex 75 10/30 HR column (GE healthcare, Buckinghamshire, UK) to confirm that all three components were in a ternary complex. The Mg-ATPase activity was measured in the presence of 0.05 mg/mL (0.092 μM) rabbit

myosin, 0.025 mg/mL (0.59 μ M) rabbit F-actin, 0.0125 mg/mL (0.19 μ M) lobster Tm, and 0.19 μ M reconstituted Tn. The assays were performed at 25°C. The other conditions were the same as described previously [17].

2.5. Fluorescence measurement

Fluorescence spectra were measured at 25°C with an excitation wavelength of 280 nm using a 650-10 Fluorescence spectrophotometer (Hitachi, Tokyo, Japan) in a solution containing 10 μ M TnC, 150 mM KCl, 20 mM MOPS-KOH (pH 6.8), 0.5 mM EGTA, 1.25 mM NTA (nitrilotriacetic acid) and either 0, 2, or 10 mM MgCl₂. The samples (1 mL) were titrated by sequential addition of 0.5 mM EGTA and 1.25 mM NTA containing 5, 50, or 200 mM CaCl₂. The volumes of titrant required to attain the desired free Ca²⁺ concentrations were calculated with a PCA calculator [20]. The Hill equation in the form:

$$\theta = \frac{[\text{Ca}^{2+}]^{nH}}{(K_{Ca}^{-nH} + [\text{Ca}^{2+}]^{nH})}$$

where θ is the relative change of the fluorescence intensity and nH is the Hill coefficient, was fitted to the data by a non-linear least squares regression method with Origin Ver. 7E software (Origin Lab, Northampton, MA, USA).

2.6. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) was performed at 25°C on a VP-ITC calorimeter (GE healthcare). TnC was dialyzed against an ITC buffer containing 0.15 M KCl, 10 mM PIPES-KOH (pH 6.8), 1 mM 2-mercaptoethanol, and either 0, 2, or 10 mM MgCl₂. The samples in a 1.4 mL cell were titrated with the ITC buffer containing CaCl₂. The sample concentrations were 210 μ M for TnC-1 (-IV) and TnC-2a (-IV), and 100 μ M for TnC-1 (-II) and TnC-2a (-II). For each titration, 15 μ L aliquots of the titrant were sequentially injected 17 times into the cell. The heat observed during the titration was analyzed using the “one set of sites” mode of the Origin-ITC analysis package (GE healthcare), and the stoichiometry, K_{Ca} , enthalpy change (ΔH), and entropy term ($T\Delta S$) of Ca²⁺-binding were estimated.

3. Results

3.1. Ca²⁺-binding properties of sites II and IV in TnC isoforms

All of the recombinant TnC proteins contain two Tyr residues at positions 95 and 102 of the Ca²⁺-deficient sites III in the C-terminal domains (Fig. 1A). Thus we attempted to detect structural change of the C-terminal domain using Tyr-fluorescence as an indicator. Large Ca²⁺-dependent changes of fluorescence were observed for TnC-1 (WT), TnC-2a (WT), TnC-1 (-II), and TnC-2a (-II), but not for TnC-1 (-IV) and TnC-2a (-IV) (Fig. 2A), indicating that the

fluorescence changes were dependent upon Ca^{2+} binding to site IV. The relative changes in the fluorescence were plotted against free Ca^{2+} concentrations, and the Hill equation was fitted to the data to estimate the K_{Ca} of site IV in each TnC (Fig. 2B and Table 1). The K_{Ca} of TnC-1 (-II) was $8.2 \times 10^5 \text{ M}^{-1}$, about one order of magnitude larger than that of TnC-2a (-II) ($6.0 \times 10^4 \text{ M}^{-1}$). The amino acid sequences of the Ca^{2+} -binding loops of sites IV for TnC-1 and TnC-2a are identical except for Ile/Leu substitution at the 8th position (Fig. 1A), suggesting that the large difference of the K_{Ca} values should be attributable to substitutions in other part of the sequences, such as the loop of site III which is known to form a β -sheet structure with that of site IV. These K_{Ca} values were virtually identical with those of corresponding wild type TnC, suggesting that the Ca^{2+} -affinity of site IV is not affected by the Ca^{2+} occupancy state of N-terminal site II. Similar assays performed in the presence of 2 or 10 mM MgCl_2 , resulted in almost the same binding curves, indicating that site IV of lobster TnC specifically binds Ca^{2+} , but is not competitively inhibited by Mg^{2+} .

Subsequently, we performed ITC analysis to determine the Ca^{2+} -binding properties of site II in the N-terminal domain. In the absence of Mg^{2+} , Ca^{2+} -binding to TnC-1 (-IV) was exothermic, while that to TnC-2a (-IV) was endothermic and entropically driven (Fig. 3A, B and Table 2). The estimated K_{Ca} values were similar for both isoforms and about $4 \times 10^3 \text{ M}^{-1}$. When Mg^{2+} concentrations were raised, the ΔH value for TnC-1 (-IV) became positive, whereas the ΔH value for TnC-2a (-IV) remained unchanged (Table 2). As a result, in the presence of 10 mM Mg^{2+} , the K_{Ca} values for site II of both isoforms significantly decreased, resulting in a K_{app} (apparent K_{Ca} in the presence of Mg^{2+}) of about 700 to 900 M^{-1} . Therefore, site II of these isoforms appears to bind Mg^{2+} competitively. The K_{Mg} values calculated from the equation:

$$K_{Mg} = \frac{(K_{Ca} - K_{app})}{K_{app} [\text{Mg}^{2+}]}$$

where $[\text{Mg}^{2+}]$ is approximated by total Mg^{2+} concentration (2 or 10 mM), were about 400 to 500 M^{-1} (Table 2).

We also performed ITC analysis of Ca^{2+} -binding to site IV of TnC-1 (-II) and TnC-2a (-II) (Fig. 3C, D and Table 2). As a result, the K_{Ca} values were rather smaller than those determined by fluorescence analysis. In addition, the K_{Ca} slightly decreased with increasing Mg^{2+} concentration, although the calculated K_{Mg} values were apparently smaller than those for site II.

Accordingly, we concluded that site II of these TnC isoforms is a low affinity Ca^{2+} -binding site, which can also bind Mg^{2+} weakly, whereas site IV specifically binds Ca^{2+} with higher affinity.

3.2. Regulatory roles of Ca^{2+} -binding to the sites II and IV

To evaluate the physiological roles of Ca^{2+} -binding to sites II and IV of lobster TnC, we tested the function of reconstituted Tn containing the mutant isoforms. The reconstituted Tn containing TnC-1 (WT) regulated the Mg-ATPase activity of actomyosin-Tm in a biphasic manner

depending on Ca^{2+} concentration, while the Tn containing TnC-2a (WT) showed apparently monophasic regulation (Fig. 4). The Tn reconstituted with either TnC-1 (-II) or TnC-2a (-II) also showed Ca^{2+} -dependent increase of the ATPase to about 29 or 47%, respectively, of the wild type Tn, indicating that Ca^{2+} -binding to site IV in the C-terminal domain plays a role in regulation. Interestingly, the ATPase activity increased with the same Ca^{2+} -dependent profile as wild type counterparts in low Ca^{2+} concentrations and reached a plateau at pCa 6.25 (TnC-1 (-II)) or pCa 5.75 (TnC-2a (-II)), probably reflecting the difference in site IV K_{Ca} values for these isoforms. In addition, the plateau activities were also different and about 0.05 and 0.09 $\mu\text{mol}/\text{mg}/\text{min}$ for TnC-1 (-II) and TnC-2a (-II), probably due to the difference in the interactions of these isoforms with TnI/TnT. These observations indicated that regulation by wild type Tn at relatively low Ca^{2+} concentrations is achieved by Ca^{2+} binding to site IV alone without a requirement for Ca^{2+} binding at site II. However, Ca^{2+} binding at site II should be essential for the maximum activity at higher Ca^{2+} concentrations, although this requires precedent Ca^{2+} binding to site IV since Tn reconstituted with TnC-1 (-IV) or TnC-2a (-IV) did not show any Ca^{2+} -dependent regulation (Fig. 4).

4. Discussion

In this report, we demonstrated the Ca^{2+} -binding properties of sites II and IV of TnC isoforms from American lobster. Site II of both TnC-1 and TnC-2a binds Ca^{2+} with the same K_{Ca} of $4 \times 10^3 \text{ M}^{-1}$ and also binds Mg^{2+} weakly with a K_{Mg} of 400 to 500 M^{-1} . The Mg^{2+} -occupancy calculated from the K_{Mg} with the assumption of the presence of 1 mM Mg^{2+} is about 0.3, indicating that most of these sites are in an apo-state in a relaxing muscle cell. The K_{Ca} values are smaller than those for the N-terminal regulatory Ca^{2+} -binding sites of vertebrate TnC ($\sim 10^5 \text{ M}^{-1}$ [3]) and might not allow intracellular Ca^{2+} binding even in the contracting state, however, the Ca^{2+} -affinities should be increased when these TnC proteins are in the thin-filament. In fact, the K_{Ca} for crayfish TnC was reported to increase about 200 times when in complex with TnI or TnI-TnT [7]. On the other hand, site IV specifically binds Ca^{2+} with a higher affinity than site II, suggesting that sites IV and II should be sequentially occupied by Ca^{2+} when the intracellular Ca^{2+} concentration is raised by neural stimulation.

We have revealed for the first time that Ca^{2+} binding to site IV as well as site II of lobster TnC participates in regulation. The ATPase-pCa curves for TnC-1 (WT) and TnC-2a (WT) differed, probably due to differences in site IV K_{Ca} values for these isoforms. In addition, each TnC will interact with one of the five isoforms of TnI *in vivo* [19], which may have different properties. Therefore, regulation mediated by two sites with different Ca^{2+} -binding profiles on the Tn complex constituted with various isoforms should confer an advantage by giving rise to complexity and variation in the shape of the tension-depolarization curve of muscle fibers. Although the abdominal flexor muscle is known to be uniformly composed of fast type fibers [21], it contains different fiber

bundles, which run transversally in each somite, longitudinally in the center, and joins different somites obliquely. Therefore, it is possible that TnC-isoforms are expressed differentially in these bundles and give distinct contracting profiles.

The molecular mechanism of site IV-mediated regulation may be similar to other Tn proteins that bind only one Ca^{2+} at site IV of TnC. TnC-F1 of insect flight muscle, which binds a single Ca^{2+} at site IV, shows Ca^{2+} -dependent regulation of binding of myosin-S1 to actin in the reconstituted thin filament [22], though TnC-F1 is known to play a role in contraction induced by the mechanical stretch of muscle fibers rather than by Ca^{2+} [14]. In addition, molluscan TnC activates the ATPase by Ca^{2+} -binding to site IV, which is accompanied by strengthening of the interaction between the C-terminal domain of TnC and the N-terminal region of TnI [17,23]. Therefore, the molecular mechanism of regulating contraction through Ca^{2+} binding to site IV of TnC may be ubiquitous in invertebrate muscles. However, it should be noted that molluscan Tn confers Ca^{2+} -sensitivity to the ATPase activity of actomyosin-Tm by strong activation in the presence of Ca^{2+} [15,17], whereas lobster Tn acts by strong inhibition in the absence of Ca^{2+} [18]. Therefore, further investigations are required to assess the similarity of these mechanisms. Our results suggest that site IV-mediated regulation is not affected by the Ca^{2+} occupancy state of site II. On the contrary, it appears likely that site II-mediated regulation requires the presence of Ca^{2+} at site IV. Thus, the structural rigidity of the 'IT-arm' [5], achieved by the strong interaction between the divalent cation bound C-terminal domain and the N-terminal region of TnI, should be important for site II-mediated mechanisms.

Thus in lobster Tn, two different regulatory mechanisms can be utilized, depending on the intracellular Ca^{2+} concentrations: a site II-mediated mechanism similar to vertebrate Tn and a novel, invertebrate-specific site IV-mediated mechanism.

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Figure legends

Fig. 1. Schematic representation of TnC mutants used in this study. A, comparison of amino acid sequences of lobster TnC-isoforms [9]. The residues identical to those of TnC-1 are represented by dots. The six Ca^{2+} -coordinating positions in the loops composed of twelve amino acids (boxed) are represented by x to -z. B, the Ca^{2+} -bindings site II or IV (open circles) in wild type TnC were inactivated by a single amino acid substitution, Glu to Gln, at the -z position of each Ca^{2+} -binding loop. C, SDS-PAGE of mutant proteins.

Fig. 2. Fluorescence titrations of TnC mutants with Ca^{2+} . A, Ca^{2+} -dependent changes of the Tyr-fluorescence for the TnC mutants were monitored in the absence of Mg^{2+} with excitation and emission wavelengths at 280 and 310 nm, respectively. B, The relative fluorescence changes for TnC-1 (-II) and TnC-2a (-II) were plotted against pCa. Triangles represent the value in the absence of Mg^{2+} . Solid, dashed, and dotted lines indicate the best fits of the Hill equation to the data in the presence of 0, 2, and 10 mM Mg^{2+} , respectively. Symbols are as follows: TnC-1 (WT) (\circ); TnC-1 (-II) (\triangle); TnC-1 (-VI) (\square); TnC-2a (WT) (\bullet); TnC-2a (-II) (\blacktriangle); TnC-2a (-IV) (\blacksquare).

Fig. 3. ITC analysis of Ca^{2+} -binding to TnC-1 (-IV) (A), TnC-2a (-IV) (B), TnC-1 (-II) (C), or TnC-2a (-II) (D). Upper panels represent the heat recorded during the titration. Lower panels show the integrated heat plotted against the molar ratio of added Ca^{2+} to TnC. Solid curves in the lower panels indicate the best fits of “one set of sites” binding model to the data.

Fig. 4. Ca^{2+} -dependent regulation of Mg-ATPase activity by Tn reconstituted with mutants of TnC-1 (A) and TnC-2a (B). The Tn reconstituted with TnC-1 (WT) (\circ), TnC-1 (-II) (\triangle), TnC-1 (-IV) (\square), TnC-2a (WT) (\bullet), TnC-2a (-II) (\blacktriangle), and TnC-2a (-IV) (\blacksquare) were added to rabbit actomyosin-lobster tropomyosin, and the Mg-ATPase activities were measured under various pCa conditions. The data are represented as means \pm s.d. of two (TnC-1 (-IV)) or three (the others) independent experiments. The ATPase activity in the absence of Tn was 0.151 $\mu\text{mol Pi/mg myosin/min}$ (\diamond).

Table 1 Summary of the Ca^{2+} -binding parameters of lobster TnC isoforms determined by Tyr-fluorescence titration.

Protein	$[\text{Mg}^{2+}]$ (mM)	K_{Ca} or K_{app} ^a (M^{-1})	nH ^b
TnC-1 (WT)	0	8.3×10^5	0.94
	2	7.6×10^5	0.83
	10	7.8×10^5	0.92
TnC-1 (-II)	0	8.2×10^5	0.91
	2	7.5×10^5	0.83
	10	8.1×10^5	0.97
TnC-2a (WT)	0	4.9×10^4	0.76
	2	4.8×10^4	0.81
	10	5.5×10^4	0.84
TnC-2a (-II)	0	6.0×10^4	0.76
	2	6.1×10^4	0.81
	10	7.5×10^4	0.77

^a Apparent K_{Ca} in the presence of Mg^{2+} .

^b Hill coefficient.

Table 2 Summary of the Ca²⁺-binding parameters of lobster TnC isoforms determined by ITC.

Protein	[Mg ²⁺] (mM)	K_{Ca} or K_{app} (M ⁻¹)	n^a	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	K_{Mg} (M ⁻¹)
TnC-1 (-IV)	0	4.2×10^3	0.97	-1.7	3.2	
	2	n. d. ^b	n. d.	n. d.	n. d.	n. d.
	10	7.1×10^2	1 ^c	1.9	5.8	4.9×10^2
TnC-1 (-II)	0	3.6×10^5	0.74	-10.3	-2.7	
	2	3.0×10^5	0.78	-9.4	-2.0	1.0×10^2
	10	1.9×10^5	0.85	-8.7	-1.4	89
TnC-2a (-IV)	0	4.1×10^3	0.78	2.6	7.5	
	2	2.3×10^3	0.83	3.3	7.9	3.9×10^2
	10	8.7×10^2	1 ^c	2.7	6.7	3.7×10^2
TnC-2a (-II)	0	2.2×10^4	0.60	-8.9	-3.0	
	2	2.0×10^4	0.64	-6.9	-1.0	50
	10	1.2×10^4	0.57	-5.7	-0.1	83

^a Stoichiometry of binding (Ca²⁺/TnC).

^b Not determined because the heat of reaction was too small to analyze.

^c Fixed to 1 to obtain the convergence of curve fitting.

A

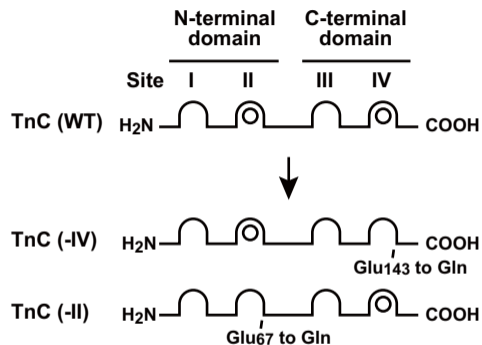
TnC-1 MDTLDEDQVQALQKAFNSFD^{x y z-y-x -z}DTDDKGFITPDTVGVILRMMGVKISDRHLQ⁵⁰EVISET
 TnC-2a ..S...E.IG.....D...S.....E.....EKN.....A..
 TnC-2b ..S..AE.LS.....D...S.....E.....EKN.....

Site I

TnC-1 DEDGSGEIEFEEF^{x y z-y-x -z}FAALAAKFLSEED⁷⁵E^{x y z-y-x -z}EALKKELKEAFRIYDRGGNGYITVHTLKE
 TnC-2aL.....VE.....I.....T.R...V..KE.....TDV...
 TnC-2bL.....VE.....I.....A.R...V.....TDV...

Site II**Site III**

TnC-1 ILREL¹²⁵DNKLTEDNLD^{x y z-y-x -z}SIIEEV¹⁵⁰DE^{x y z-y-x -z}DGSGTIDFNEFMKMMNG
 TnC-2aR...ED.....L.....E...
 TnC-2bR...ED.....L.....Q...

Site IV**B****C****Fig. 1 (2-column fitting)**

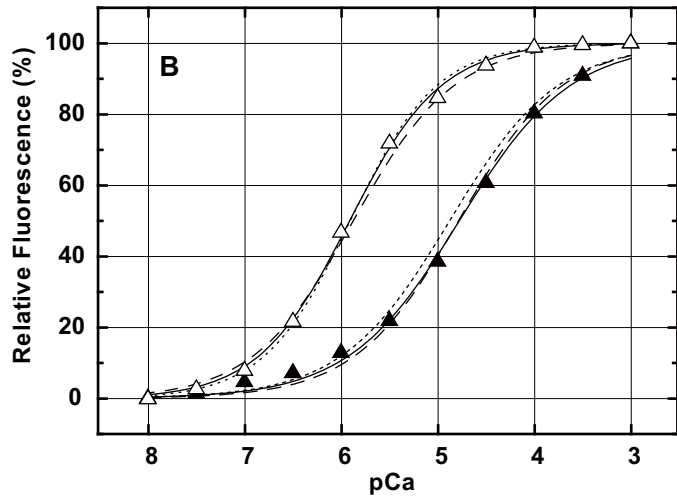
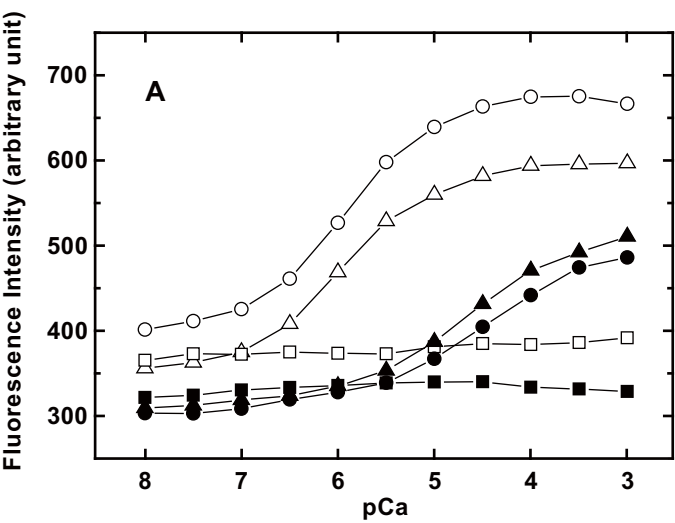


Fig. 2 (2-column fitting)

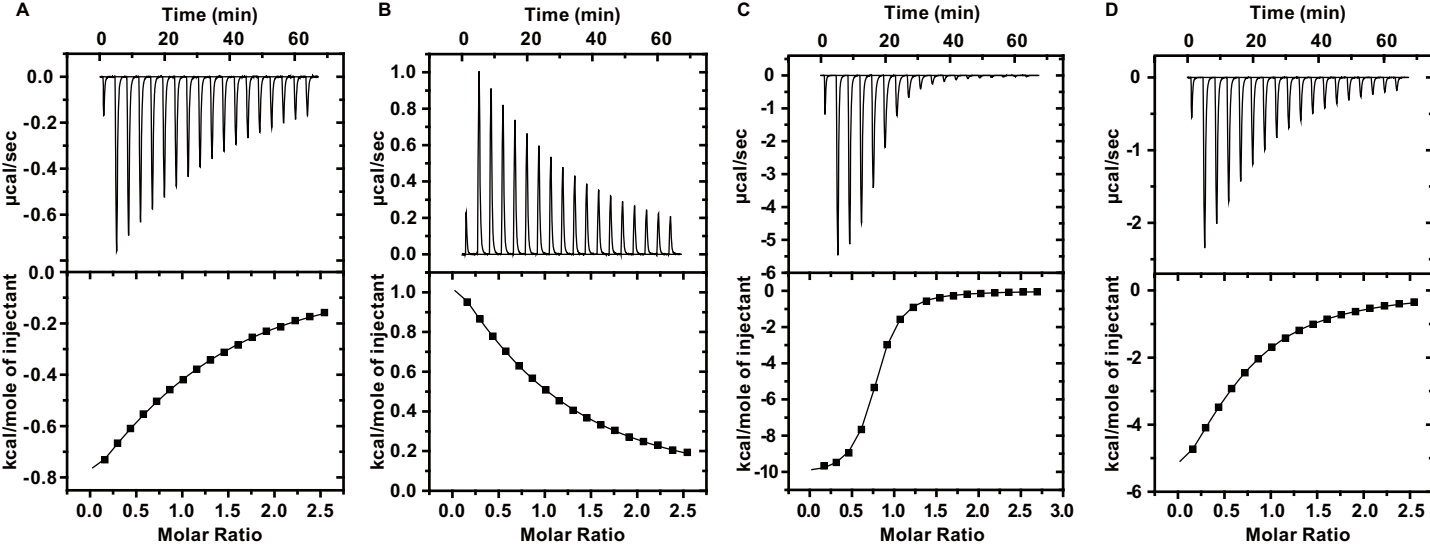


Fig. 3 (2-column fitting)

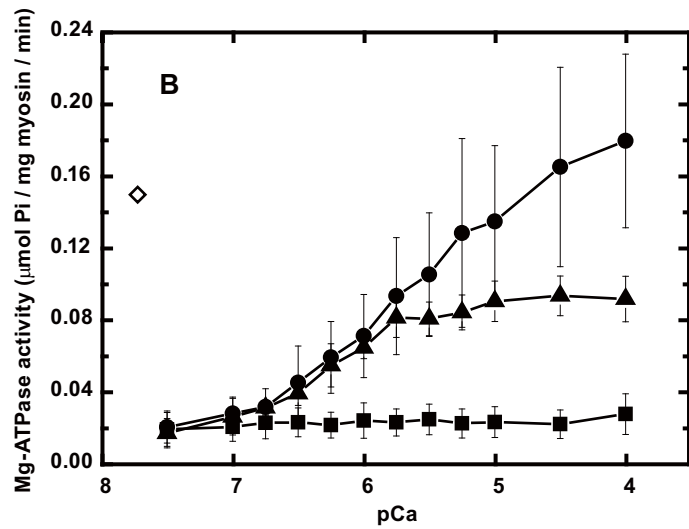
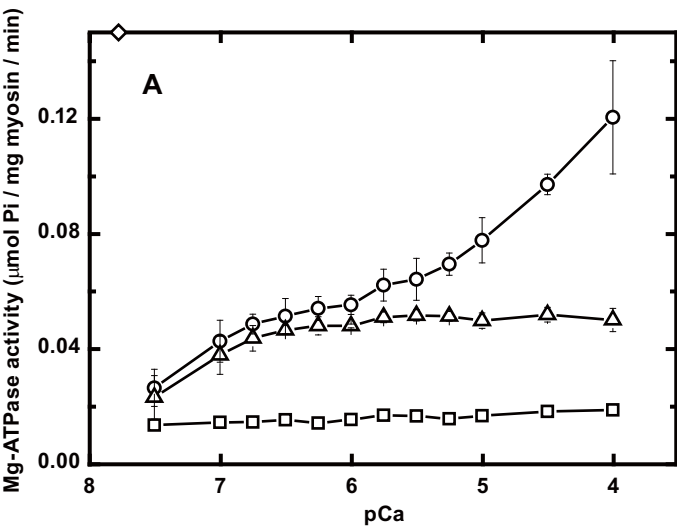


Fig. 4 (2-column fitting)