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Effect of N-terminal extension of cardiac troponin I on the Ca^{2+} regulation of ATP-binding and ADP dissociation of myosin II in native cardiac myofibrils

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Abstract

Cardiac troponin I (cTnI) has a unique N-terminal extension that plays a role in modifying the calcium regulation of cardiac muscle contraction. Restrictive cleavage of the N-terminal extension of cTnI occurs under stress conditions as a physiological adaptation. Recent studies have shown that in comparison with controls, transgenic mouse cardiac myofibrils containing cTnI lacking the N-terminal extension (cTnI-ND) had lower sensitivity to calcium activation of ATPase, resulting in enhanced ventricular relaxation and cardiac function. To investigate which step(s) of the ATPase cycle is regulated by the N-terminal extension of cTnI, here we studied the calcium dependence of cardiac myosin II ATPase kinetics in isolated cardiac myofibrils. ATP binding and ADP dissociation rates were measured by using stopped flow spectrofluorimetry with mant-dATP and mant-dADP, respectively. We found that the second order mant-dATP binding rate of cTnI-ND mouse cardiac myofibrils was three-fold as fast as that of wild type myofibrils in low Ca^{2+} . The ADP dissociation rate of cTnI-ND myofibrils was positively dependent on calcium concentrations while the wild type controls were not significantly affected. These data from experiments using native cardiac myofibrils under physiological conditions indicate that modification of the N-terminal extension of cTnI plays a role in the calcium regulation of the kinetics of actomyosin ATPase.

Graphical Abstract

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CONFLICT OF INTEREST DISCLOSURE

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SUPPORTING INFORMATION AVAILABLE

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Troponin (Tn) is a thin filament regulatory protein in striated muscle, controlling contraction and relaxation through regulating the cross-bridge cycling between sarcomeric myosin II and actin filaments in response to intracellular calcium concentration^{1–4}. The troponin complex is composed of three protein subunits^{4, 5}: the calcium-binding subunit troponin C (TnC), the tropomyosin-binding subunit troponin T (TnT), and the actomyosin ATPase inhibitory subunit troponin I (TnI).

Three homologous genes are present in vertebrates encoding two skeletal muscle isoforms and one cardiac isoform of TnI^{6–9}. While sharing the highly conserved core structure with the skeletal muscle isoforms, cardiac TnI (cTnI) has a unique N-terminal extension of ~30 amino acids containing two adjacent serine residues (Ser-23/Ser-24) that are substrates for protein kinase A (PKA) phosphorylation^{6, 10, 11}. cTnI also contains a calcium dependent regulatory region and a coiled-coil region (the I–T arm) that interacts with TnT and transmits calcium signals in the troponin complex^{12–15}. Recent studies have suggested that the N-terminal extension of cTnI regulates the function of cTnI through long range conformational effect on the I–T interface^{15, 16}.

The functional importance of the N-terminal extension of cTnI in cardiac muscle contractility has been intensively studied^{2, 17–19}. Upon β -adrenergic stimulation, phosphorylation of Ser-23/Ser-24 by PKA facilitates cardiac muscle relaxation^{20, 21}. Selective removal of the N-terminal extension by restrictive proteolysis naturally occurs *in vivo* in hemodynamic stress adaptations^{22, 23}. Interestingly, removal of the N-terminal extension mimics the effects of PKA phosphorylation as a compensation for β -adrenergic deficiency. Transgenic mouse hearts expressing cTnI lacking the N-terminal extension (cTnI-ND) showed decreased Ca^{2+} sensitivity, and enhanced ventricular relaxation¹⁷. Echocardiographic measurements demonstrated that the hearts of aging cTnI-ND transgenic mice (16-month-old) exhibited better systolic and diastolic functions than age-matched wild type controls²⁴.

At the myofilament level, muscle contraction is initiated by the binding of Ca^{2+} to TnC, which subsequently leads to changes in troponin conformation and movement of tropomyosin away from myosin binding sites of actin filaments, and the activation of actomyosin cross-bridge cycling^{4, 25}. The cooperative process of the cross-bridge binding kinetics is important in the cardiac muscle as a determinant of systolic and diastolic functions²⁶. Thin filaments in muscle are turned off at low calcium concentration and are turned on at high calcium concentration. How does N-terminal extension of cTnI regulate the on/off system? Despite its effect on regulating the relaxation kinetics of cardiac muscle,

the molecular mechanism for the N-terminal extension of cTnI to regulate the cross bridge cycling of myofilaments is not fully understood.

The N-terminal extension of cTnI has been reported to regulate the calcium sensitivity of the cross-bridge cycle and decreased the steady state ATPase activity of myofibrils, which partially accounts for the enhanced relaxation and improved cardiac function^{4, 8, 9, 13, 16, 17}. However, how the cTnI N-terminal extension affects the individual step of the actomyosin cross-bridge cycle remains to be investigated.

To understand the ATPase cycle of actomyosin, transient kinetics and mechanical force measurements have been studied with actomyosin S1^{27–39}, myofibrils^{40–49}, and muscle fibers^{48, 50, 51}. Extensive studies have been done with actomyosin subfragment-1 (S1) and -heavy meromyosin (HMM). Lymn and Taylor pioneered measurement of the steps of ATPase cycle by using stopped flow spectrofluorimetry^{52, 53}. Bagshaw and Trentham then reported the ATP binding rate measured using intrinsic tryptophan fluorescence using stopped flow spectrofluorimetry⁵⁴, showing an increase with dissociation of actomyosin and ATP cleavage. Others also characterized the ATPase cycle using intrinsic protein fluorescence^{43, 47, 55–57} and fluorescence nucleotide analogues^{32, 58, 59}. These kinetic studies have identified the steps of ATPase cycle (Scheme 1)⁶⁰. Quench-flow technique was also employed to measure the actomyosin kinetics and has a higher time-resolution than stopped flow spectrofluorimetry^{40, 42, 58, 61, 62}. One ATP analogue, mant-dATP, has been used to determine the rate in ATPase steps with myosin or actomyosin^{28, 63, 64} and myofibrils^{28, 40, 65}. The advantage of using mant-dATP is that its change in fluorescence when binding to the ATP binding site of the myosin motor domain is larger (~2.5 fold) than the changes in intrinsic fluorescence of the myosin and that the emission spectra of mant fluorescence does not overlap with that of tryptophan.

The ATPase cycle of actomyosin consists of the following steps: the equilibrium of initial actomyosin-ATP complex (constant K_1), the myosin head isomerization step (k_2'), ATP hydrolysis step (k_3), phosphate release (k_4'), and ADP release (k_5') (Scheme 1). The calcium dependent kinetic mechanism of the myofibril and regulated actomyosin ATPase have been extensively studied^{33, 39, 40, 66–68}. While the rate constants for ATP binding and ADP dissociation for unloaded myofibrils is approximately that of regulated acto-S1, they showed no significant calcium dependence. However, the rate constant for the phosphate burst transient is larger in the presence of calcium as opposed to that in the absence of calcium, which is also in agreement with regulated acto-S1^{39, 40}. These studies suggest that while that ATP binding and ADP dissociation events are not regulated by calcium.

The benefit for utilizing such powerful kinetic studies is that we are able to identify the individual rates of the ATPase cycle (e.g., ATP binding, ATP hydrolysis, Pi release, and ADP dissociation). Experiments using purified actomyosin are able to control the concentrations of and the ratio between thin filament and myosin. A disadvantage of purified actomyosin system is the lack of thin filament regulatory system thus making this system less representative for physiological conditions. Thus, this study used purified myofibril system of cTnI-ND mutant comparing wildtype (WT) myofibrils. To examine the role of cTnI N-terminal extension in regulating actomyosin kinetics, we measured the ATP binding

and ADP dissociation rates of myosin II in transgenic mouse cardiac myofibrils that contain solely cTnI-ND under low and high calcium concentrations. The results demonstrated that the ATP binding of cTnI-ND myofibrils was three times as fast as that of wild type (WT) myofibrils containing intact cTnI in low Ca^{2+} but exhibited no difference in high Ca^{2+} . The fast ADP dissociation rate for cTnI-ND myofibrils was positively dependent on $[\text{Ca}^{2+}]$ while that of WT myofibrils was $[\text{Ca}^{2+}]$ independent. These data provide evidence that the N-terminal extension of cTnI regulates the Ca^{2+} -responsiveness of the ATP binding and ADP dissociation kinetics of actomyosin cross-bridge cycle, whereas the calcium activation of ATPase in myofibrils does not fully correlate with the rates of ATP binding and ADP dissociation.

EXPERIMENTAL PROCEDURES

Reagents and mouse heart samples

Mant-dADP (2'-Deoxy-3'-O-(N'-methylantraniloyl) adenosine-5'-O-diphosphate) and mant-dATP (2'-Deoxy-3'-O-(N'-methylantraniloyl) adenosine-5'-O-triphosphate) were purchased from Axxora (Farmingdale, NY). Use of C57BL/6 mice was conducted under the guidelines of protocols approved by the Wayne State University Animal Care and Use Committee. Double transgenic mice expressing cTnI-ND on the background of endogenous cTnI gene-deleted were described previously.⁶⁹ Wild type mice of the same strain (C57BL/6) were used as control. Ventricles of 2–3 month-old WT and cTnI-ND mice⁹ were rapidly removed after euthanasia and rinsed with ice-cold phosphate-buffered saline (PBS). The heart tissues were snap-frozen in liquid nitrogen and kept at -80°C for a short period before storage in liquid nitrogen. Our goal in this study is also to determine whether myofibrils isolated from the frozen heart can be used as a model in a solution kinetics study to determine the function of N-terminal extension of cTnI for ATPase cycle.

Preparation of myofibrils from frozen cardiac muscle

Cardiac myofibrils were prepared using a modified method as previously described^{33, 40, 44, 70}. Briefly, the frozen heart was thawed on ice and minced into small pieces, followed by homogenization in ice-cold rigor buffer (10 mM Tris-HCl pH 7.0, 5 mM EGTA, 130 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1 mM NaN_3 , 1 mM DTT, 0.1 mM PMSF, 1 μM Papstatin A and 1 $\mu\text{g}/\text{mL}$ Leupeptin) with an electric homogenizer at 9,000 rpm for 10 sec (IKA T25, Model: T25DS, Fisher Scientific, Pittsburgh, PA) and cooled down on ice for 30 sec three times. The homogenate was then centrifuged at 2,500 $\times g$, 4°C for 5 minutes, and the supernatant was removed. The pellet was suspended with washing solution buffer (60 mM KCl, 30 mM imidazole, pH 7.0, 2 mM MgCl_2 , 1 mM DTT, 1 $\mu\text{g}/\text{mL}$ Leupeptin and 0.5% v/v Triton X-100) and centrifuged at 2,500 $\times g$ again for 5 minutes. Three more washes were repeated using the same buffer without Triton X-100. The pellets were re-suspended in a buffer containing 20 mM MOPS pH 7.0, 1 mM EGTA, 5 mM MgCl_2 , 100 mM KCl. The suspension was filtered with a mesh net (70 μm pore-size, Fisher Scientific), centrifuged at (2,500 $\times g$, 4°C for 2 min), and kept on ice for use within a few days. Myofibrils were also prepared from freshly dissected mouse hearts using the same procedure to validate the use of frozen mouse hearts. The concentration of myofibrils was estimated from UV absorbance at 280 nm. We used the extinction coefficient 7.0, which was previously determined by Sutoh

et al, after dissolving the myofibril by adding sodium dodecyl sulfate to the suspension⁷¹. Myosin concentration was calculated as 50% of the total myofibrillar protein^{10, 33, 71}.

SDS-PAGE, Western-blotting, Pro-Q staining

SDS-PAGE and Western-blotting were performed as previously described^{22, 69}. The details are provided in the Supporting Materials. Pro-Q staining was performed as previously described²².

Steady state ATPase activity

Steady state ATPase activity was measured using an NADH-coupled ATP-regenerating system. The details are provided in the Supporting Materials.

Imaging of purified myofibrils

Myofibrils from frozen and fresh cTnI-ND and WT mouse hearts were imaged using differential interference contrast (DIC) and fluorescence confocal microscopy using a Zeiss, Axio Plan2, LSM 510 META microscope with Plan-NEOFLUAR 63x/1.25 oil lens. Details of the method are described in the Supporting Materials.

Measurements of solution transient kinetics

All transient kinetic measurements of myosin's intrinsic tryptophan (Trp) fluorescence were performed with a stopped flow spectrofluorimetry apparatus (SFM-300, Biologic USA, Knoxville, TN) using a micro-cuvette (μ Sf-8, cuvette volume = 4 μ l) operated by the provided software (Bio-kine, Bio-logic USA).

The suspension of myofibrils and the solution of ADP and ATP analogues were prepared in Buffer A (20 mM MOPS pH 7.0, 1 mM EGTA, 5 mM $MgCl_2$, 100 mM KCl with various concentrations of Ca^{2+}). The experimental temperature (23°C) was controlled by a water-chiller system (Endocal RET series, Thermo Fisher Scientific, Waltham, MA). The dead-times were 0.25 msec for a total flow-rate of 13.3 ml/s and 1 msec for a total flow-rate of 4 ml/s. Mant-dATP and mant-dADP were excited at 280 nm wavelength via fluorescence resonance energy transfer (FRET) from an intrinsic tryptophan residue (Trp⁵⁰⁹) in the motor domain of cardiac myosin II. The emitted light was selected using a 400 nm long pass filter, and the fluorescence signal was acquired at 90° from the excitation path.

To measure the rate of mant-dATP binding to myosin in the myofibrils, single mixing reaction was performed. Myofibril suspension (1 μ M head concentration) in syringes 1 and 2 and various concentrations of mant-dATP solution in syringe 3 were mixed with a two-to-one ratio for myofibrils and mant-dATP, respectively, and the final myosin head concentration in the cuvette was 0.67 μ M. The suspension and solution were prepared in Buffer A in the absence of ATP. Calcium was added at appropriate concentrations.

To measure the rates of ADP and mant-dADP dissociation from myosin, double mixing reactions were performed. Myofibrils, mant-dADP, and ADP were prepared in Buffer A, as described above. Myofibril suspension (1 μ M myosin head concentration) in syringe 1 and 2 μ M mant-dADP or ADP in syringe 2 were mixed with a one-to-one ratio and incubated for

20 sec in a delay line. Then, the solution was mixed at a one-to-one ratio with excess ADP (1 mM) in syringe 3 for mant-dADP dissociation experiment or with 800 μ M mant-dADP for ADP dissociation experiment. The final myosin head concentration in the cuvette was 0.67 μ M.

The fitting of experimental data to the time course was done using software provided by Bio-logic USA (Bio-kine) and Origin Pro Ver 8.5 (Origin lab, Northampton, MA). Each datum point (i.e., at a given pCa and a given nucleotide concentration) was averaged over 4 ~ 6 traces and was fitted to a single (Eq. 1 for less than 50 μ M nucleotide) or double (Eq. 2 for nucleotide concentrations $\geq$ 50 μ M) exponential equation for mant-dATP binding experiments and double exponential equation for ADP and mant-dADP dissociation experiment as follows:

$$I(t) = I_0 \exp(-k_{\text{Fast}} t) + C \quad (\text{Eq. 1})$$

$$I(t) = I_1 \exp(-k_{\text{Fast}} t) + I_2 \exp(-k_{\text{Slow}} t) + C \quad (\text{Eq. 2})$$

where $I(t)$ is the fluorescence signal over the time t , and k_{Fast} and k_{Slow} are the apparent rates. I_0 , I_1 , and I_2 are the corresponding amplitude coefficients of the fast and slow exponential parts. The fitting goodness was evaluated by the r^2 value in order to determine whether the data can be fitted by a single or double exponential.

Statistical analysis

All data are presented as mean $\pm$ S.D., and n refers to the number of mouse hearts studied. Statistical analysis for the difference between the cTnI-ND and WT groups was performed using paired two-tailed Student's t -test and significance is reported as $p < 0.05$ and $p < 0.01$.

RESULTS

Preparation of active cardiac myofibrils from frozen adult mouse hearts

To assess the integrity of isolated myofibrils, the protein extracts from intact heart tissues and myofibrils were analyzed using SDS-PAGE and Western blotting (Fig. 1 A). As shown in Fig. 1A, SDS-PAGE showed that the representative myofibrillar proteins such as myosin heavy chain, actin, cTnI and cTnI-ND were preserved in the isolated myofibrils (Fig. 1A top). Quantitative densitometry of SDS-PAGE gels found no significant difference of myosin and actin between the total cardiac muscle homogenate and isolated myofibrils (data not shown). Immunoblot analysis using anti-TnI monoclonal antibody TnI-1 confirmed the sole presence of cTnI-ND in the transgenic mouse cardiac myofibrils and the absence of intact cTnI (Fig. 1A, bottom). The data demonstrated the effectiveness of preparing adult cardiac myofibrils from frozen mouse hearts for functional studies. Note that the phosphorylation level of myosin light chain (MLC) and myosin binding protein C (MBP-C) in cTnI-ND and WT was analyzed by Pro-Q staining, and the level of both proteins was not difference (Fig. S1 A and B).

To demonstrate that the purified myofibrils preserved sarcomeric structure, myofibrils from frozen and fresh hearts were labeled with rhodamine phalloidin and imaged by a confocal microscope (see Fig. S2 in the Supporting Material) in the presence and absence of ATP and without calcium. Fluorescence images were used to measure the intensity of the position of the thin filament by line-scan (Fig. S2, red). The intensities were then analyzed by the sum of Gaussian function (Fig. S2 C, F, L, and O). The distance between peaks was calculated and plotted as a histogram (Fig. S2 G, H, P, and Q), which was fit with a single Gaussian function (Fig. S2 G, H, P, and Q). The sarcomere lengths of cTnI-ND and WT with and without ATP were not significantly different ($\sim 1.8 \mu\text{m}$) under all conditions (Table S1 in the Supporting Material).

Thickness (width) of myofibrils was measured by DIC images with the line scan method. The parameters obtained from all groups were not significantly different ($1.1 \sim 1.3 \mu\text{m}$, Table S1). No shortening of myofibrils was found.

cTnI-ND results in decreased sensitivity of cardiac myofibril actomyosin ATPase to calcium activation

The steady-state ATPase activity of myofibrils prepared from frozen and fresh hearts of cTnI-ND and WT mice was measured under different Ca^{2+} concentrations (pCa 3–9). For frozen heart experiments, the maximal ATPase rate (V_{max}) of cTnI-ND myofibrils (red open circle, $10.1 \pm 0.42 \text{ s}^{-1}$) ($< \text{pCa} 4$) was slightly lower than that of WT (open circle, $12.7 \pm 0.77 \text{ s}^{-1}$). The ATPase rates of WT and cTnI-ND at pCa 9 were 0.36 ± 0.12 and $0.29 \pm 0.09 \text{ (s}^{-1}\text{)}$, respectively. The pCa_{50} of ATPase activation for frozen heart were 5.32 ± 0.12 for cTnI-ND and 6.29 ± 0.08 for WT myofibrils, respectively ($p < 0.01$ for comparing the rates of 50% maximum ATPase, Fig. 1B), demonstrating a lower calcium sensitivity of the cTnI-ND myofibrils as compared with that of WT myofibrils. The steady state ATPase activity of myofibrils from fresh (non-frozen) mouse hearts (cTnI-ND: black circle, WT: blue circle) further validated the use of frozen heart for reliable kinetics studies (Fig. 1B).

N-terminal extension of cTnI affects Ca^{2+} response of the initial actomyosin-ATP complex formation

To evaluate the ATP-binding rates, myofibrils were mixed with various amounts of mant-dATP at pCa 9, 6, and 3, and the fluorescence intensity was monitored over time. Single mixing stopped flow spectrofluorimetry was used to observe the mant-dATP binding event (Fig. 2). The data at $20 \mu\text{M}$ mant-dATP were well fit with a single exponential function while the data at higher mant-dATP concentrations were well fit with a double exponential function as determined by the r^2 values.

After the flow was stopped at 4 msec in Fig. 2B and 2C, the increasing fluorescence signals were analyzed. To confirm whether the increasing signals were ATP binding events, we examined the same experiment in the absence of myofibrils, the absence of mant-dATP, and the absence of both mant-dATP and myofibrils (Fig. S3 A and B in the Supporting Material). In the absence of myofibrils, the signals did not increase at all mant-dATP concentrations after stopped flow, indicating that the changes were solely from mant-dATP binding to myosin. Note that the changing of mant-dATP signal was detected before the syringe

stopped. Thus, the signal at the point of stopping the syringe is proportional to mant-dATP concentration. In the absence of mant-dATP or/and myofibrils, the fluorescence signals before and after syringe stop were not changed, meaning that the fluorescence signal from tryptophan or mant-dATP itself did not leak out from the emission filter (long pass filter from 400 nm, Fig S2 C and D). Recent studies showed that the rate of kinetics was detected by a conformational change in the Trp residue when ATP binds to myosin. To confirm that our data after the flow stopped did not include these Trp signal, myofibril suspension and 1 mM ATP was mixed and monitored (Fig S2 E). The fluorescent signal from Trp was not detected before and after stopped flow. This shows that the 400 nm long pass filter cut out the Trp signal. Further confirming that the increased signal after the stopped flow resulted from the mant-dATP binding events.

The fast and slow phases for all ATP concentrations were plotted in Fig. 3. In Fig. 3A–C, the data were fitted by Michaelis-Menten. The K_1 (mM^{-1}) is calculated by $1/K_m$ (K_m (mM), which is the [ATP] at the half maximum rate), and k_2' is the maximum rate.

k_2' (s^{-1}), which represents the myosin head isomerization step (Scheme 1), was not significantly affected by calcium concentration in both cTnI-ND and WT myofibrils (Fig. 3A – C and Table 1). However, k_2' (s^{-1}) was consistently higher in cTnI-ND myofibrils than that of WT under all pCa conditions examined.

During the fast rate of ATP-binding, we found that K_1 (mM^{-1}), which is the equilibrium constant for the formation of the initial actomyosin-ATP complex in the myosin ATPase kinetic cycle (Scheme 1), was higher at pCa 6 than that at pCa 9 and 3 in WT myofibrils. However, K_1 of cTnI-ND myofibrils was not different between pCa 9 and 6 but decreased three fold in pCa 3 (Table 1).

K_1k_2' ($\mu\text{M}^{-1}\text{s}^{-1}$), which is the second order-rate constant of mant-dATP binding and represents the initial slope at low concentration of mant-dATP ($< 20 \mu\text{M}$) (Scheme 1), of cTnI-ND myofibrils was ~ 3.4 fold as fast as that of WT myofibrils at pCa 9 ($p < 0.01$). The difference in K_1k_2' ($\mu\text{M}^{-1}\text{s}^{-1}$) between cTnI-ND and WT myofibrils was diminished when Ca^{2+} concentration was increased (Table 1 and Fig. 3 D – F, $p < 0.01$ for pCa 3 vs. pCa 9 and $p < 0.01$ for pCa 3 vs. pCa 6). A ~ 2.5 fold higher K_1k_2' was observed in WT myofibrils at pCa6 than that at pCa 3 and 9 (both $p < 0.01$; pCa 3 vs. pCa 6 and pCa 6 vs. pCa 9), which was not seen in cTnI-ND myofibrils.

In contrast to the fast ATP-binding rates, the slow rates of ATP binding obtained from the double exponential fitting at higher concentrations of mant-dATP ($> 50 \mu\text{M}$) were not significantly different between cTnI-ND and WT myofibrils under the Ca^{2+} concentration examined (Fig. 3 G – I). The slow rates were independent of mant-dATP concentration and were around $25 \sim 40 \text{ s}^{-1}$. The amplitudes of fast and slow rates of the total intensity after stopped syringes were 86% and 14%, respectively, and were independent of ATP concentrations. Note that the student's t test value of cTnI-ND at 100 mM at pCa 9 is only $p > 0.05$. There is only 18 % difference between cTnI-ND and WT. Although it is difficult to interpret this result in contrast to that in purified actomyosin-S1 experiments, recent studies suggested that a slow rate step in such an experimental setting should be the ATP hydrolysis

$(k_3+k_{-3})^{43}$. Actomyosin S1 studies found that the ATP cleavage rate (k_3+k_{-3}) is about 10–30% of k_2' ³⁷. Thus, our slow mant-dATP binding rate may indicate the ATP hydrolysis rate.

cTnI-ND decreases the rate of mant-dADP dissociation at low Ca^{2+}

We next measured the rates of mant-dADP dissociation from myosin in the cTnI-ND and WT myofibrils at various Ca^{2+} concentrations (pCa 9 through 3) using a double-mixing method by the addition of excess ADP (1 mM) to a pre-mixed solution containing 2 μM mant-dADP and 1 μM myofibrils (Fig. 4 A through F). The decreased fluorescence signal induced by mant-dADP dissociating from myosin was fitted to a double exponential equation (Eq. 2, Fig. 4 A through F), while the single exponential function did not fit the data well. The corresponding fast and slow rates of mant-dADP dissociation were determined (Fig. 4 G, H, and Table 2).

The fast rate of mant-dADP dissociation in cTnI-ND myofibrils was $250 \pm 36 \text{ s}^{-1}$, much slower than that of WT myofibrils at pCa 9 (396 s^{-1} , $p < 0.01$). However, the dissociation rate of mant-dADP in cTnI-ND myofibrils was increased to 429 s^{-1} when increasing the Ca^{2+} concentration to pCa 3 ($p < 0.01$, Fig. 4D and Table 2). By contrast, the dissociation rate of mant-dADP in WT myofibrils was not significantly different between pCa 9 and pCa 3 ($p > 0.05$, Fig. 4 D and Table 2).

To confirm the mant-dADP dissociation rates obtained from ADP binding, we also examined the dissociation of ADP from myofibril actomyosin under various Ca^{2+} concentrations (pCa 9 through 3) using mant-dADP chasing method in which excess mant-dADP (800 μM) was added to the premixed solution containing 1 μM myofibril and ADP. The fluorescence intensity increased over time due to mant-dADP association to cardiac myosin II (Fig. S5 in the Supporting Materials). Consistent with the results in Fig. 4, the fast rate of ADP dissociation in cTnI-ND myofibrils at pCa 9 was lower than that of WT myofibrils (Fig. S3 D, $290 \pm 41 \text{ s}^{-1}$ for cTnI-ND vs. 426 ± 38 for WT, $p < 0.01$) but was dramatically increased when increasing Ca^{2+} concentration to pCa 3 ($499 \pm 39 \text{ s}^{-1}$, $p < 0.001$ for comparing the rates between pCa 9 and pCa 3), while the rate of WT myofibrils was not significantly affected by the change of calcium concentrations ($p > 0.07$ for comparing ADP dissociation from WT between pCa 9 and pCa 3).

cTnI-ND did not affect the slow rates of mant-dADP and ADP dissociation

The slow rates of mant-dADP and ADP dissociation were calculated from data shown in Fig. 4 A–F and Fig. S5 A–F in the Supporting Material, respectively. The rates were not significantly different between the cTnI-ND and WT myofibrils under all Ca^{2+} concentrations tested, and both were slightly decreased when Ca^{2+} concentration was increased. The amplitude of the slow rates was less than 5% of the total amplitude of the fluorescence intensity, suggesting that the slow rate is due to a small fraction of exchange between ADP binding and dissociation.

DISCUSSION

Assessing ATPase activity in native myofibrils

cTnI differs from the fast and slow skeletal muscle isoforms of TnI mainly by the N-terminal extension^{7, 72}. The embryonic heart expresses solely slow skeletal muscle TnI that ceases expression during postnatal development while cTnI up-regulates to become the sole TnI in adult hearts⁷. Therefore, the N-terminal extension of cTnI is an adult heart-specific regulatory structure.

To investigate the mechanistic basis for the regulatory function of the N-terminal extension of cTnI, we studied the ATPase kinetics of myofibrils isolated from cTnI-ND and WT mouse hearts. ATPase assay at various calcium concentrations confirmed that cTnI-ND myofibrils have lower calcium sensitivity than the WT controls. Previous studies showed that the N-terminal truncated cTnI mimics the effect of phosphorylation or pseudo-phosphorylation mutations of Ser^{23/24} on decreasing the calcium affinity of troponin, resulting in faster relaxation of cardiac muscle^{10, 17, 18, 24, 73}. Our results obtained from isolated myofibrils are consistent with the previous observation, confirming that the calcium sensitivity of cTnI-ND myofibril ATPase is lower than that of WT control.

The data that myofibrils prepared from snap frozen mouse hearts could be effectively used in the transient kinetics studies to quantitatively measure the ATP binding and ADP dissociation rates significantly facilitates the application of long term stored muscle tissues for functional investigations.

A previous rapid quench-flow experiment showed that the ATPase of myofibrils was 50 to 100 times activated by calcium⁵⁸. Although, our myofibril ATPase assay was taken more than one second, the results showed about 35 fold increases from pCa 9 to 3. This slightly lower activation may be due to the fact that the high calcium concentration at pCa 4 caused myofibrils to over contract and not to be able to produce optimal activity.

The N-terminal extension of cTnI does affect ATP binding at pCa 9, but does not affect ATP binding to cardiac myosin II

Myofibril ATPase assay at different calcium concentrations was measured and activated 10-fold from pCa 9 to pCa 3 (Fig. 1 B). Mant-dATP binding rate from pCa 9 and pCa 3 did not show such large difference (Fig. 3 A–C, Table 1). Thus, we conclude that mant-dATP binding did not significantly contribute to the ATPase over the range of calcium concentrations. However, at pCa 9, the K_{1k_2} rate of cTnI-ND is three times faster than that of WT. To understand this result, we need to examine the calcium sensitivity of the troponin complex for actomyosin interaction. The troponin complex is a regulatory protein, integral for interaction between actin and myosin. It regulates the “on” and “off” state between actin and myosin. Without the N-terminal extension, cTnI has a decreased calcium sensitivity, which suggests that the off state is more stable against calcium concentration. In this case, the N-terminal extension plays an important part for controlling the on and off state. Typically WT myofibrils would reside in the off state at low calcium concentrations, but our data suggests that there exists a small probability that the WT myofibrils can fluctuate between the on and off state. This fluctuation could possibly be due to the thermal

instabilities within the myofibril at room temperature. Therefore, since cTnI-ND can retain a more stable off state than that of WT at pCa 9, more myosin heads are exposed, allowing for a faster ATP binding rate. Thus, the ATP binding rate of cTnI is three time faster than that of WT.

To further understand the meaning of fluorescence changes in FRET signals from mant-dATP binding events for cTnI-ND and WT myofibrils, it is important to analyze the absolute initial intensity signals and absolute fluorescence changes (Fig. S3). When the flow was stopped, the fluorescence signal of mant-dATP with myofibrils can be measured as the initial fluorescence signal. The amplitude of the signal corresponds to the amount of mant-dATP initially binding to myosin in the myofibrils. Note that some mant-dATP fluorescence signal leaked from the 400 nm long pass filter and the initial intensity is proportional to the increasing mant-dATP concentration (Fig. S3 A and B). Thus, the initial fluorescence signal was subtracted by the mant-dATP fluorescence signal alone (Fig. S3 B) as an absolute initial signal (Fig. S4 A – C). The absolute initial intensities of both cTnI-ND and WT myofibrils are proportional to the increase in mant-dATP concentration and saturated after 300 μ M. The absolute fluorescence change is the total fluorescence signal change after the flow was stopped (Fig. S4 B). The absolute fluorescence changes of cTnI-ND at pCa 9 and 6 are similar for all mant-dATP concentrations, but the absolute fluorescence changes at pCa 3 increased more at higher mant-dATP concentrations. This result shows similar behavior to the results of the ATPase assay (Figure 1B), but there was not enough difference between pCa 6 and pCa 3 ATPase rates. The plots of the absolute fluorescence change for WT myofibrils were similar in manner at all pCa. These results showed a little calcium sensitivity for cTnI-ND myofibril signals.

The ratio between absolute fluorescence change and absolute initial intensity was plotted against pCa (Fig. S3 C). At pCa 9 and 2 μ M mant-dATP, the ratio between the fluorescence signal change and initial intensity (Fig. S3 C) is much higher than that of WT. It is also important to note that mant-dATP easily binds to myosin in cTnI-ND myofibrils in this condition.

N-terminal extension of cTnI controls Ca^{2+} -dependent ADP dissociation

We employed double-mixing stopped flow methods to investigate the effect of the N-terminal truncated cTnI on ADP dissociation from cardiac myosin in WT and cTnI-ND myofibrils from pCa 9 to pCa 3. Together with the mant-dADP chasing results in Fig. S5, the amplitudes of the slow rates were independent of pCa and were ~5% of total signal after the syringes stopped. Thus, we conclude that the data represent true ADP dissociation events.

Our results in Fig. 4, Fig. S5, and Table 2 showed that the fast rates of ADP and mant-dADP dissociation from cTnI-ND myofibrils are calcium dependent, whereas the rates of WT myofibrils are faster and did not significantly change over calcium concentrations. As we discussed above about the on and off rate sensitivity for calcium, this is also a similar phenomenon in which N-terminal deletion of cTnI can block the mechanical characteristics of the troponin complex, while WT cTnI may work via a weak transition between on and off state at lower calcium concentrations. Thus, pCa dependence of the fast rate of ADP

dissociation from WT myofibrils can be explained by the calcium sensitivity of myofibril state.

Previous studies have shown that the ADP dissociation rate from actomyosin is ~100-fold faster than that from myosin alone, meaning that thin filament binding to myosin accelerates ADP dissociation^{32, 37, 40, 74, 75}. Thus, the ADP dissociation rate depends on the number of cross-bridges of actomyosin. Our results suggest that at low calcium concentrations corresponding to that in a relaxed cardiomyocyte, myosin heads in cTnI-ND myofibrils may bind less to the thin filament than that in WT myofibrils.

The rate-limiting step in actomyosin ATPase cycle can be changed by tension in the myofilaments. For isometric contraction of muscles, the ADP release step is the rate-limiting step, whereas in shortening and force reducing contractile muscle fibers, the Pi release is the rate-limiting step. Recently, an attached and detached pathway model⁴⁴ was proposed to explain muscle contraction, which was based on the Geeves and Holmes model^{30, 76, 77}. The Lionne and Barman model suggests that Pi release in myofibrils is the rate-limiting step because of the reduced force in the myofibrils^{44, 78}, in which myofibrils represent a system of “attached pathway”. In cTnI-ND myofibrils myosin may bind the thin filament less than that in WT myofibrils at pCa 6 due to the lower calcium sensitivity of troponin, resulting in less myosin heads bound to the thin filaments and the accumulated AM\ADP-Pi state. To test this hypothesis, phosphate release measurements are needed in future studies.

Transient kinetics studies are a powerful tool that can measure each step in the myosin ATPase in myofibrils, thus determining which step limits the myosin ATPase. These results are useful to explain the differences in ATPase rates of WT and cTnI-ND, which govern changes in the thin filament regulation of contraction and relaxation in the myofibril. In this study, we determined the ATP binding and ADP dissociation rates in which these rates did not significantly contribute to the calcium sensitivity of myofibril ATPase from pCa 9 to 3.

Based on the experiments using native mouse cardiac myofibrils in the presence of physiologically integrated myofilaments, our present study demonstrated that the N-terminal extension of cTnI plays a role in regulating the actomyosin ATPase cycle of the cardiac muscle. More studies are needed to fully understand how the restrictive proteolysis regulation of the adult heart-specific N-terminal extension of cTnI^{17, 22, 23} functions in the calcium activation and kinetics of each step of the cardiac myosin II ATPase cycle. Although our data showed that the ATP binding rates and ADP dissociation rates did not have large contributions to the calcium regulation of ATPase in isolated myofibrils due to the unloaded condition, the slower and calcium dependent fast rates of ADP dissociation from cTnI-ND myofibrils (Fig. 4, Fig. S5, and Table 2), implicate a function of the N-terminal extension of cTnI in regulating the relaxation of cardiac muscle as that observed in ejecting working hearts^{17, 22}. Therefore, the rate limiting effect of the phosphate release step⁴⁴ merits further investigation.

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ABBREVIATIONS

cTnI	cardiac troponin I
cTnI-ND	cardiac troponin I lacking the amino terminal extension

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FIGURE 1. Myofilament protein composition and steady state ATPase activities of myofibrils isolated from cTnI-ND transgenic and WT mouse hearts

(A) Top-panel: The SDS-PAGE gel shows the preserved myofilament protein composition in myofibrils isolated from frozen WT and cTnI-ND transgenic mouse hearts in comparison with that of fresh intact cardiac muscle. Bottom-panel: Western blot using anti-TnI monoclonal antibody TnI-1 confirmed the presence of solely cTnI-ND in the cTnI-ND transgenic hearts and myofibrils. MHC: myosin heavy chain. (B) ATPase activity of cTnI-ND ($n = 5$ mice, open red; frozen heart and $n = 4$ mice, closed black; non-frozen heart) and WT ($n = 5$ mice, open black circle; frozen heart and $n = 4$ closed blue; non-frozen heart) cardiac myofibrils in the absence of and the presence of various calcium concentrations (pCa) at 23 °C. The pCa_{50} values of myofibril for frozen and non-frozen hearts were 5.32 ± 0.12 , 5.21 ± 0.13 for cTnI-ND and 6.29 ± 0.08 , 6.25 ± 0.15 for WT, respectively ($p < 0.01$, in Student's t test for ATPase rate between cTnI-ND and WT at pCa 6).

FIGURE 2. Fluorescence traces of mant-dATP binding to cardiac myosin in cTnI-ND and WT myofibrils

Typical traces of mant-dATP binding to isolated myofibrils at pCa 6. Isolated myofibrils were mixed with various concentrations of mant-dATP at different Ca^{2+} concentrations (pCa 6). Binding events were monitored by FRET signal via intrinsically conserved tryptophan 509 to mant-dATP at 23 °C (see Materials and Methods). (A – C) Normalized typical traces of mant-dATP binding for cTnI-ND myofibrils (D–F) and for WT myofibrils (D–F) at pCa 6. Note that the maximum signal from at 800 μM mant-dATP was used to normalize all data. Three different mant-dATP concentrations (5 μM for A and B, 50 μM for B and E, and 500 μM for C and F) are shown. The data were fit with single exponential function for A ($r^2 = 0.952$) and D ($r^2 = 0.861$) and double exponential function for B ($r^2 = 0.863$), C ($r^2 = 0.901$), E ($r^2 = 0.855$), and F ($r^2 = 0.851$). at pCa 6. All conditions used four hearts of cTnI-ND ($n = 4$) and WT ($n = 4$). Three to four traces at each data point were averaged. The averaged data from three to four heart-sets was calculated as mean $\pm$ S.D. All insets in A – F are short time scales (100 msec).

FIGURE 3. Fast and slow rates of mant-dATP binding rate of cardiac myosin

(A – C) The analysis of the fast rate obtained from the fluorescence traces of mant-dATP binding for cTnI-ND ($n = 4$ hearts, closed circle) and WT ($n = 4$ hearts, open circle) myofibrils against different ATP concentrations at different Ca^{2+} concentrations (A pCa 9; B, pCa 6; and C, pCa 3). Data were fitted by Michaelis-Menten. (D – F) The second order rate constant (K_1k_2') of mant-dATP binding to myosin in the myofibrils was calculated at lower mant-dATP concentration ($< 20 \mu\text{M}$). The p value for comparing the K_1k_2' rates between cTnI-ND and WT myofibrils were: D, $p < 0.01$; E, $p < 0.05$; F, $p > 0.07$. Data are fitted with a linear regression. (G – I) The slow rate of mant-dATP binding rates. The rates for cTnI-ND (open circle) and WT (closed circle) myofibrils were measured at three different pCa (9, 6, and 3). Solid lines (cTnI-ND) and dot lines (WT) are the average of all data sets. Student's t tests for all data against the average were performed ($p < 0.05$). Data represent means $\pm$ S.D. All experiments performed at 23°C .

FIGURE 4. Fluorescence traces of mant-dADP dissociation at different calcium concentrations (A –F)) Double mixing reactions were performed to measure the rate of mant-dADP dissociation from cardiac myosin in the myofibrils at 23 °C. 1 μ M myofibril and 2 μ M mant-dADP were mixed and incubated for 20 sec in a delay line and then the solution was mixed with 1 mM ADP. Typical traces of mant-dADP dissociation from cTnI-ND and WT myofibrils with different pCAs were shown. Inset is the data until 0.05 sec. The trace shown was the averaged with three different traces. The data were fitted with double-exponential equation (red line). (G) The fast phase of mant-dADP dissociation rates in cTnI-ND (closed circle) and WT (open circle) myofibrils. Data were fitted with non-linear least-squares regression. Asterisks indicate the statistical significances in Student's *t* sets. * $p < 0.01$ of cTnI-ND myofibrils for comparing the rates between pCa 3 and pCa 9. ** $p > 0.05$ WT myofibrils for comparing the rates between pCa 3 and pCa 9. # $p < 0.01$: comparing cTnI-ND and WT at pCa9. ## $p = 0.06$: comparing cTnI-ND and WT at pCa9. (H) The slow phase of mant-dADP dissociation for cTnI-ND and WT myofibrils. Data for cTnI-ND (solid line) and WT (dashed line) myofibrils were fitted with non-linear least-squares regression. Data represent means $\pm$ S.D.

Scheme 1.

Table 1

Parameter of mant-dATP binding experiments

measurement	Parameter	cTnl-ND myofibrils			WT myofibrils			
		pCa	9	6	3	9	6	3
mant-dATP binding	K ₁ (mM ⁻¹)		26.3 ± 1.6	27.8 ± 2.2	8.1 ± 1.1	17.9 ± 6.0	27.8 ± 2.1	10.9 ± 8.1
	k ₂ [′] (s ⁻¹)		228 ± 32	195 ± 11	200 ± 19	98 ± 18	140 ± 16	128 ± 19
	K ₁ k ₂ [′] (μM ⁻¹ s ⁻¹)		6.00 ± 0.21 ^{a,c}	5.42 ± 0.41 ^b	1.62 ± 0.11 ^{a,b}	1.75 ± 0.21 ^{c,d}	3.89 ± 0.22 ^{d,e}	1.40 ± 0.33 ^e
	k ₃ + k ₋₃ (s ⁻¹)		38.5 ± 8.5 ^f	32.9 ± 4.3 ^g	34.3 ± 4.4 ^h	26.8 ± 4.3 ^f	25.6 ± 4.5 ^g	25.6 ± 5.9 ^h

All experiments used four hearts (n = 4) and a single mixing method at 23 °C (see Methods section).

Values are mean ± S.D. Means and S.D. were calculated by four averaged data at each heart set (e.g. cTnl-ND and WT set) at different days.

a and b $p < 0.01$ for comparing the $K_1 K_2'$ rates of cTnl-ND between pCa 3 and 9 and between pCa 3 and 6, respectively

c $p < 0.01$ for comparing the $K_1 K_2'$ rates at pCa 9 between cTnl-ND and WT

d and e $p < 0.01$ for comparing the $K_1 K_2'$ rates of WT between pCa 6 and 9 and between pCa 3 and 6, respectively.

f, g, and h $p > 0.05$ for comparing the $k_3 + k_{-3}$ of cTnl-ND and WT at each pCa

Table 2

Parameter of mant-dADP and ADP dissociation

Measurement	cTnl-ND myofibrils					WT myofibrils					
	pCa	9	7.5	6	4.5	3	9	7.5	6	4.5	3
mant-dADP dissociation	250±36 ^{a,c}	270±20.3	324±40	356±38	386±38 ^a	396±32 ^{b,c}	366±27	366±43	333±43	349±28 ^b	
ADP dissociation	290±41 ^{d,f}	353±38	455±32	431±13	499±39 ^d	426±38 ^{e,f}	466±50	391±35	397±42	383±50 ^e	

All experiments used a double mixing method at 23 °C (see Materials and Methods). Values are mean +/- S.D.

- ^a p < 0.01 for comparing mant-dADP dissociation from cTnl-ND between pCa 9 and pCa 3.
- ^b p > 0.05 for comparing mant-dADP dissociation from WT between pCa 9 and 3.
- ^c p < 0.01 for comparing mant-dADP dissociation at pCa 9 between cTnl-ND and WT.
- ^d p < 0.001 for comparing ADP dissociation from cTnl-ND between pCa 9 and pCa 3.
- ^e p > 0.07 for comparing ADP dissociation from WT between pCa 9 and pCa 3.
- ^f p < 0.01 for comparing ADP dissociation at pCa 9 between cTnl-ND and WT.