Structural and Functional Significance of Aspartic Acid 89 of the Troponin C Central Helix in Ca^{2+} Signaling†

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Received July 19, 1996; Revised Manuscript Received October 1, 1996⊗

ABSTRACT: The central helix of troponin C is highly conserved in length and amino acid sequence. In this region, D89 is conserved and specific to TnC. To investigate its significance, three mutations were made in avian fast troponin C: (1) D89 was replaced with A (D89A); (2) the central helix was replaced with a designed α-helix (αh89A) consisting of ^{87}AEAALKAAMEA^{97}; and (3) A89 of αh89A was replaced with D (αh89D). D89A and αh89A activated the regulated actomyosin ATPase poorly in the presence of Ca^{2+} (24 ± 1.0% and 14 ± 2.0%, respectively, of the wild type maximal activity) whereas αh89D had higher activity (113 ± 3%). Both αh89A and D89A had apparently normal interactions with TnI and TnT whereas αh89D formed a complex with TnT even in the absence of Ca^{2+}. The central helix was also replaced with a flexible random coil and rigid polyproline linkers in which D89 was Arg or Pro, respectively. Like αh89A and D89A, both mutants were defective in activation of the actomyosin ATPase in the presence of Ca^{2+}. Changes in regulatory function of the mutants did not correlate with altered Ca^{2+} affinity, altered conformational changes upon binding divalent cations, or Ca^{2+}-dependent binding to TnI or TnT. The results suggest that D89 is required for Ca^{2+}-dependent signal transduction, an event that can be dissociated from Ca^{2+}-dependent binding to TnC targets on the thin filament.

Troponin C (TnC)[1] is a Ca^{2+}-binding protein that, in association with the other regulatory components of the thin filament, troponin I (TnI), troponin T (TnT), and tropomyosin (TM), is involved in the transmission of Ca^{2+} signals for striated muscle contraction (Grabarek et al., 1992; Farah & Reinach, 1995; Tobacman, 1996). The crystal structure of TnC is an elongated dumbbell shaped molecule, consisting of two Ca^{2+}-binding domains connected by a nine turn α-helix. Three turns in the middle of this linker region, the central helix (the D/E linker), are exposed to solvent (Herzberg & James, 1988; Satyshur et al., 1988). The D/E linker is poorly ordered in the NMR solution structure (Slupsky & Sykes, 1995). The structure of TnC is similar to that of calmodulin, the major differences being that CaM has a shorter helix at the extreme N-terminus, and the D/E linker is shorter, lacking ^{91}KGK^{93} (Babu et al., 1988). The length and sequence requirements of the central helix have been extensively investigated and will not be reviewed here.

In the X-ray structure of TnC, in which the Ca^{2+}-specific sites are unoccupied, there are electrostatic and hydrogen bonding interactions between D89 and Q51, N52 in the BC loop and Q85 in the D helix; see Figure 1 (Herzberg & James, 1988; Satyshur et al., 1988). When Ca^{2+} binds to the two Ca^{2+}-specific regulatory sites in the N-terminal domain, the structure changes such that the BC helices move away from the AD helices, exposing a patch of hydrophobic residues, as well as Q51 and Q85 (Slupsky & Sykes, 1995; Gagné et al., 1995). Presumably, D89 also becomes more exposed, although this residue is poorly ordered in the solution structure. This conformational change is essentially as originally proposed by Herzberg et al. (1986), who suggested that the exposed hydrophobic region would provide the site for Ca^{2+}-dependent binding to TnI and TnT.

The sequence of residues 86−97 in the central helix is conserved in cardiac and striated TnC. The triplet ^{88}EDA^{90} is DTD in calmodulin. A mutation corresponding to D89A reduces the calcium sensitizing effect of the drug, levosimedan (Pollesello et al., 1994). Gulati et al. (1993) showed that TnC with ^{88}EDA^{90} converted to DTD was able to partially activate phosphodiesterase activity, mimicking calmodulin. Activation was even greater if the EDA/DTD mutation was combined with deletion of ^{91}KGK^{93}, lacking in calmodulin. The TnC function of these mutants was not investigated.

In this study, we have defined the importance of D89 for Ca^{2+} signaling by fast skeletal TnC. We constructed a series of mutants with and without D89 in the native and engineered central helices. Our results show D89 is crucial for Ca^{2+}-dependent thin filament activation irrespective of the helical environment. Mutants in which residues 87−97 were replaced with a flexible random coil or a rigid polyproline motif, where Asp89 was Arg or Pro, respectively, also failed to activate. Portions of this work have been reported in a preliminary form (Ramakrishnan et al., 1996).

† This work was supported by grants from the National Institutes of Health (GM36326) and the Muscular Dystrophy Association, and by a Postdoctoral Fellowship from the American Heart Association, New Jersey Affiliate, to S.R.

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⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1996.

[1] Abbreviations: Tn, troponin; TnC, troponin C; TnI, troponin I; TnT, troponin T; TM, tropomyosin; CaM, calmodulin; CD, circular dichroism; DTT, dithiothreitol, T_m, melting temperature.

S0006-2960(96)01788-6 CCC: $12.00

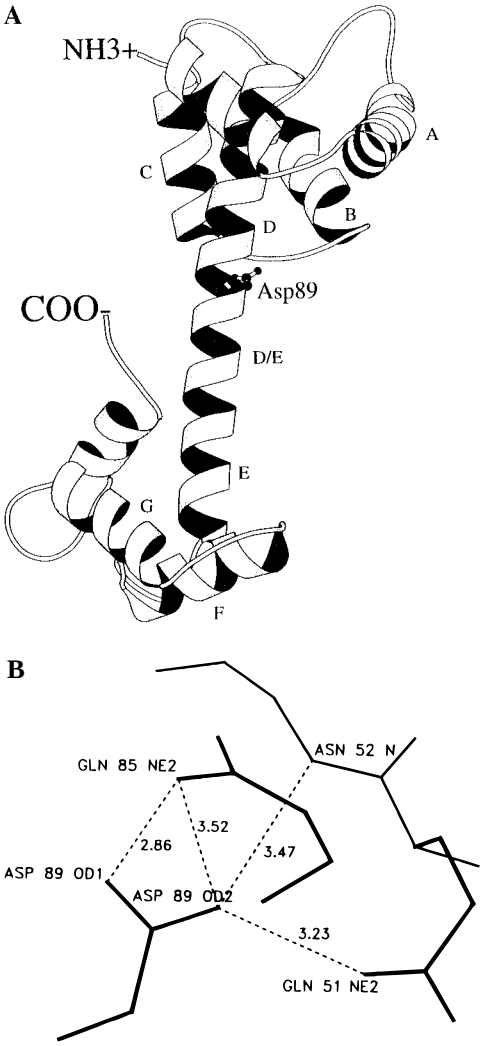

FIGURE 1: Molecular model of wild type TnC: (a) Molecular model of TnC showing the location of D89 within the tertiary structure of TnC (Herzberg & James, 1988). The model was generated using the MOLSCRIPT (Kraulis, 1991). (b) This panel shows interactions between D89 and Q51, D89 and N52 in BC loop D89 and E85 in D-helix generated by using Xtalview (Xfit) program (Evans, 1993).

MATERIALS AND METHODS

Mutant Design and Construction. A synthetic cDNA for the chicken fast skeletal muscle TnC was used in this study (Xu & Hitchcock-DeGregori, 1988). Two codons in the original synthetic cDNA sequence were changed using site-directed mutagenesis to match the amino acid sequence of chicken pectoral muscle TnC (E99A, D100N) (Golosinska et al., 1991).

The mutants were constructed using oligonucleotide-directed mutagenesis using a Muta-Gene *in vitro* mutagenesis kit (Bio-Rad; Zoller & Smith, 1983; Kunkel, 1985). The mutations were confirmed by complete sequencing of the cDNA (Sanger et al., 1977). Table 1 shows the TnC mutants made, the oligonucleotides used, and the predicted amino acid sequences of mutant TnCs.

Routine molecular biological techniques such as preparation of plasmid, M13 single, and RF DNA, agarose gel electrophoresis, restriction enzyme digestion, dephosphorylation, and ligation were performed as described by Sambrook et al. (1989) or as recommended by the supplier.

Expression and Purification of Proteins. The cDNAs were overexpressed in *Escherichia coli* BL21 (DE3) cells using pET3d or pET11d vectors (Studier et al., 1990) and purified according to previously published methods (Dobrowolski et al., 1991b; Ramakrishnan & Hitchcock-DeGregori, 1995).

Circular Dichroism (CD) Spectroscopy. (A) Far-UV CD Measurements. The far-UV CD spectrum was analyzed from 250 to 200 nm, every 0.5 nm, with 2-s collection time in a 1 or 2 mm path length rectangular quartz cell using an Aviv Model 62 DS spectropolarimeter as previously described (Ramakrishnan & Hitchcock-DeGregori, 1995). Thermal unfolding was recorded at 222 nm by increasing the temperature from 2 to 90 °C. The T_ms reported are the observed midpoint of the second derivative of the major transition.

(B) Calcium Titration. The protein samples were dialyzed against 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 1.0 mM DTT, 0.9 mM EGTA, and 0.9 mM nitrilotriacetic acid (NTA) for 48 h with two changes. Ca^{2+} titrations were carried out and analyzed as previously described (Dobrowolski et al., 1991b; Smith et al., 1994).

ATPase Measurements. The thin filament was reconstituted by mixing actin, TM, and TnIT or TnI in a 7:1.5:2.5 molar ratio in 10 mM imidazole, pH 7.0, 1 mM DTT, and 0.15 M NaCl (final concentration). Troponin−IT complex was prepared by combining TnI and TnT (in urea) in 1:1 molar ratio and dialyzing against 10 mM imidazole, pH 7.0, 0.25 M NaCl, and 1 mM DTT. The actin mixture was sedimented at 60 000 rpm, 4 °C, for 30 min (Beckman TL-100 ultracentrifuge, TL-100.2 rotor). The actin pellet was rinsed and resuspended in the same buffer. The same method was followed for preparing reconstituted thin filaments with TnI.

The actomyosin ATPase assays were performed in a total volume of 75 μL, in the conditions described in the figure legends, in a thermoequilibrated microplate reader (Molecular Devices) at 28 °C, as previously described (Ramakrishnan & Hitchcock-DeGregori, 1995). The amount of inorganic phosphate released was determined colorimetrically according to White (1982) in microtiter plates and was read with a 650 nm filter. The data were fit to the Hill equation using SigmaPlot.

Polyacrylamide Gel Electrophoresis. 8% polyacrylamide gels containing 6 M urea were used for the analysis of complex formation of TnC with TnI, and nondenaturing 8% polyacrylamide gels were used to detect the complex formation between TnC and TnT, as described by Head and Perry (1974). SDS−polyacrylamide gel electrophoresis was performed according to Laemmli (1970).

General Methods. Contractile proteins were prepared from chicken pectoral muscle. Troponin was prepared according to Potter (1982) with the modifications that protease inhibitors were added during extraction. α-Tropomyosin was purified from the isoelectric precipitate of the Tn preparation (Hitchcock-DeGregori et al., 1985). Actin was purified from an acetone powder according to Hitchcock-DeGregori et al. (1982); myosin was prepared as described by Margossian and Lowey (1982).

Troponin C concentrations were determined using a biuret assay with bovine serum albumin as a standard (Goa, 1954) and spectrophometrically determined using the extinction coefficient (A(1% at 260) = 1.2). The concentration was

Table 1: Design of Troponin C Central Helix Mutants

TnC	Amino Acid Sequence	Oligonucleotide	Comments
WT	^{87}KEDAKGKSEEE^{97}		
D89A	^{87}KEAAKGKSEEE^{97}	3′-CTTTCTTCGTCGATTTCC-5′	Asp 89 of wild type was converted to Ala
αh89A	^{87}AEAALKAAMEA^{97}	3′-CAGGCAGTCTACCGTCTCCGTCGCGACTTT CGCCGCTACCTCCGCGAACGTTTGACG-5′	wild type amino acid sequence was replaced with a designed α-helical linker[a]
αh89D	^{87}AEDALKAAMEA^{97}	3′-CCGTCTCCTGCGCGACTTTC-5′	Ala 89 of αhrep was converted to Asp
rcrep	^{87}GRGRGEGEGDG^{97}	3′-CAGGCAGTCTACCCGGCACCGGCACCGCTCC CACTCCCGCTACCAGAACGTTTGACG-5′	wild type amino acid sequence was replaced 11-residue random coil linker[b]
pprep	^{87}PPPPPP - - -^{97}	3′-CAGGCAGTCTACGGGGGTGGCGGAGGCGGAG AACGTTTGACG-5′	wild type amino acid sequence was replaced 6-residue rigid two turn polyproline (18.6Å)[c]

[a] The residues in this linker have a high propensity of forming an α-helix, and in addition, $i + 4$ interaction between the charged residues should also favor a strong α- helical conformation (Marqusee & Baldwin, 1987). [b] Glycine as every alternate residue should result in a flexible random coil structure of the linker. [c] The six-residue proline replacement should form a rigid two turn, all *trans*-polyproline II-like helix (Deber et al., 1970) without a major alteration in length and orientation between the Ca^{2+}-binding domains.

confirmed by amino acid analysis. The concentrations determined using the three methods were in good agreement, differing by <10%. Other protein concentrations were determined spectrophotometrically using the following extinction coefficients (A(1% at 280)): actin, 11.0; tropomyosin, 3.0; myosin, 5.3; Tn, 4.5; TnI, 4.0; TnT, 5.0.

RESULTS

Mutant Design. The sequence requirements of the TnC central helix (D/E linker) for function were investigated using a series of mutations shown in Table 1 that change the sequence but maintain the approximate overall length of the linker. In αh89A, the eleven residues of the exposed region of the central helix (residues 87−97) were replaced with a sequence of eleven amino acids that has a high probability to form three turns of α-helix. In the sequence, the conserved Asp at residue 89 in TnC was Ala. Possible i to $i + 4$ interactions between the charged residues E88, K92, and E96 were retained to enhance stability (Marqusee & Baldwin, 1987). To investigate the functional significance of D89 in particular, we made two "point" mutations: in D89A, Asp89 in the wild type sequence was changed to Ala; in αh89D, Ala89 in the αh89A mutant was "reverted" to Asp. All mutant proteins were well-expressed in *E. coli*.

Conformation of Mutant Troponin Cs. The secondary structure of the TnC mutants was analyzed using circular dichroism spectroscopy (Table 2). The α-helix content of all mutants increased upon binding of Mg^{2+} to the high affinity sites, with a further increase upon binding of Ca^{2+} to the low affinity sites, as previously published (Kawasaki & van Eerd, 1972; Johnson & Potter, 1978; Dobrowolski et al., 1991a; Babu et al., 1993; Smith et al., 1994; Ramakrishnan & Hitchcock-DeGregori, 1995). However, the α-helical content of αh89D was higher than the wild type in all occupancy states whereas that of αh89A was lower even though the only difference between these two mutants is residue 89 (Table 1). In addition, in αh89A the percent increase in the α-helical content upon binding Mg^{2+} was significantly lower (31%) than αh89D (52%) or wild type (48%). The T_ms of the major transitions in Mg^{2+}/EGTA were as follows: wild type, D89A, αh89A, rcrep, 70 °C; αh89D, pprep, 68 °C, indicating that the mutations do not affect the overall stability of TnC.

Table 2: α-Helical Content of Mutant TnCs Determined by Circular Dichroism Spectroscopy[a]

	mean residue ellipticity at 222 nm (% α-helix)[b,c]		
TnC	EDTA	Mg^{2+}/EGTA	Ca^{2+}
WT	−15 500 ± 1170 (39)	−20 700 ± 1100 (58)	−21 700 ± 1000 (61)
D89A	−15 500 ± 300 (40)	−20 650 ± 400 (57)	−22 500 ± 200 (64)
αh89A	−14 470 ± 1000 (36)	−17 240 ± 1100 (47)	−20 500 ± 520 (57)
αh89D	−17 600 ± 320 (46)	−24 300 ± 600 (70)	−26 300 ± 650 (76)
rcrep	−12 670 ± 750 (30)	−15 300 ± 100 (39)	−18 600 ± 700 (50)
pprep	−12 680 ± 930 (30)	−19 650 ± 850 (53)	−20 400 ± 1200 (56)

[a] The far-UV circular dichroism spectra were determined at 2 °C, in a 1 mm cuvette, 0.1−0.2 mg/mL protein. Conditions: 2 mM HEPES buffer (pH 7.0) containing 50 mM NaCl, 1 mM DTT, and either 2 mM EDTA, 5 mM $MgCl_2$ and 1 mM EDTA, or 3 mM $CaCl_2$. [b] The mean residue ellipticity was calculated according to Adler et al. (1973). The values given here are the average of three to five scans. [c] The percent α-helix (shown in parentheses) was calculated according to Greenfield and Fasman (1969).

Regulation of Actomyosin ATPase. Relief of TnIT Inhibition. Regulation of the actomyosin MgATPase in a reconstituted thin filament system was used to assay the ability of the mutants to activate the thin filament in a Ca^{2+}-dependent fashion. Figure 2a shows that αh89A was defective in the activation of actomyosin ATPase activity in the presence of $Ca^{2+}$and had only about 14 ± 2.0% ($n = 4$) of the wild type activity, although it did cosediment with the reconstituted thin filament with and without Ca^{2+} (results not shown). The critical importance of residue 89 for activation is illustrated by comparing D89A and αh89D in this assay (Figure 2b). When Asp89 was changed to Ala in wild type TnC (D89A), the activation of the actomyosin ATPase in the presence of Ca^{2+} was only 24 ± 1% ($n = 3$) of wild type, comparable to the engineered central helix (αh89A). In both mutants residue 89 is A instead of wild type D. These results indicate the absence of D at this position can significantly affect TnC regulatory function, independent of the composition of the α-helical environment. The sigmoidal shape of the curve (as seen in Figure 2b) most likely relates to the cosedimentation of excess of TnI or TnIT with the reconstituted thin filaments.

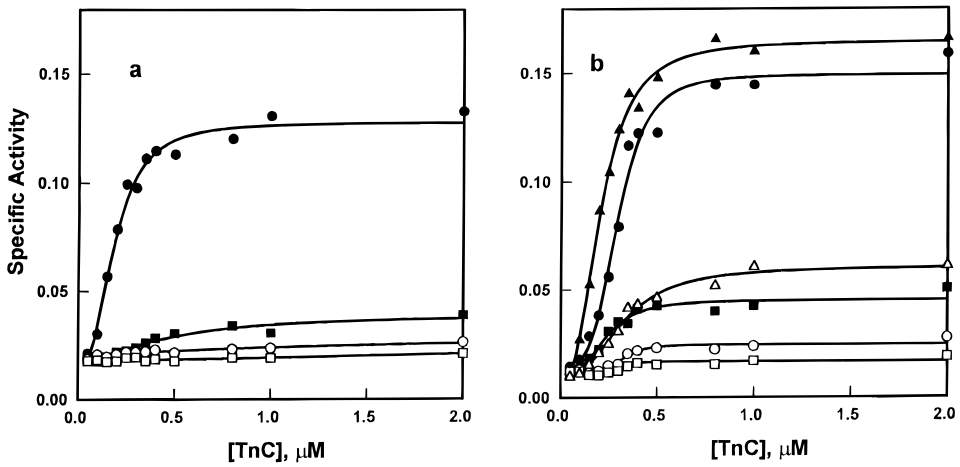

FIGURE 2: Regulation of the actomyosin ATPase activity by wild type, D89A, αh89A, and αh89D. Increasing concentrations of TnC were added to a reconstituted thin filament complex containing actin, tropomyosin, TnI, and TnT. The final concentrations were myosin (0.9 μM), thin filament (about 2.4 μM actin), and TnC (0−2 μM) in 10 mM imidazole, pH 7.0, 40 mM NaCl, 0.5 mM $MgCl_2$, 0.5 mM ATP, and 0.1 mM $CaCl_2$ or 0.2 mM EGTA. The assay was performed for 15 min at 28 °C. Specific activity is expressed as μmol of P_i/(mg of myosin·min). (a) ○, ● wild type; □, ■ αh89A. (b) ○, ● wild type; □, ■ D89A; △, ▲ αh89D. Filled symbols: 0.1 mM $CaCl_2$; open symbols: 0.2 mM EGTA.

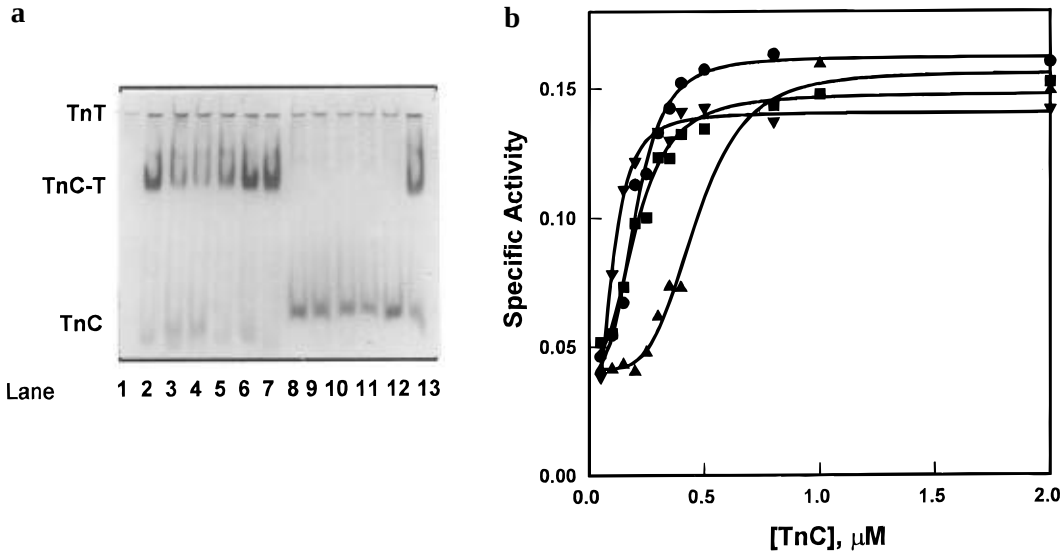

FIGURE 3: (a) Electrophoresis of TnT-C in polyacrylamide gels. TnT and TnC were combined in 2.5 M urea/10 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 2 mM $CaCl_2$ or 5 mM EGTA and analyzed on 8% polyacrylamide gels. Lanes 2 and 8, wild type; 3 and 9, αh89A; 4 and 10, rcrep; 5 and 11, pprep; 6 and 12, D89A; 7 and 13, αh89D. Lanes 2−7, $+Ca^{2+}$; lanes 8−13, $-Ca^{2+}$. (b) Relief of troponin I inhibition of the actomyosin ATPase by wild type, D89A, αh89A, and αh89D. Conditions: The assay was carried out as described in Figure 2 except the reconstituted filament contained actin, tropomyosin, and TnI (no TnT) in 0.2 mM EGTA. ● wild type; ■ D89A; ▼ αh89A; ▲ αh89D.

The functional role of D89 was further substantiated when we converted A89 of αh89A to Asp. Interestingly, this mutant, αh89D, recovered the activity completely and showed consistently higher actomyosin activity in the presence of Ca^{2+} than the wild type TnC (113 ± 1%, $n = 3$) (Figure 2b). However, the αh89D mutant partially relieved TnIT inhibition of the ATPase even in the absence of Ca^{2+}, resulting in poorer inhibition of the actomyosin ATPase, compared to wild type TnC (αh89D, 66 ± 5%, vs wild type, 88 ± 2%, $n = 3$). Our results show that D89 is crucial for the activation of regulated actomyosin ATPase activity in the presence of Ca^{2+}, irrespective of the composition of the α-helix background. The failure of complete inhibition of actomyosin ATPase activity by αh89D in the absence of Ca^{2+} also suggests that other residues in the wild type central helix may be required for inhibition (Dobrowolski et al., 1991a). All the TnCs bound to the regulated thin filament in the presence and absence of Ca^{2+}, as assayed by cosedimentation. Therefore, the failure of αh89A and D89A to activate the ATPase cannot be attributed to failure to bind.

Interaction with TnI and TnT. To investigate if the impaired regulatory function of TnC mutants is due to altered subunit interaction with TnI or TnT, we analyzed the Ca^{2+}-dependent binary complex formation of TnC with TnI and TnC with TnT in polyacrylamide gels (Head & Perry, 1974). All TnC mutants formed binary complexes with TnI or TnT in the presence of Ca^{2+} (Figure 3a; TnI results not shown), but surprisingly, the αh89D mutant formed a complex with TnT even in the absence of Ca^{2+} (lane 13, Figure 3a).

We also assayed the ability of TnC mutants to relieve the TnI inhibition of actomyosin ATPase activity in the presence and absence of Ca^{2+} using a partially reconstituted thin filament (no TnT). The relief of inhibition of actomyosin activity in the presence of Ca^{2+} by D89A, αh89A, and αh89D was comparable to wild type TnC (results not shown). Figure 3b shows that, in the absence of Ca^{2+}, all TnCs fully

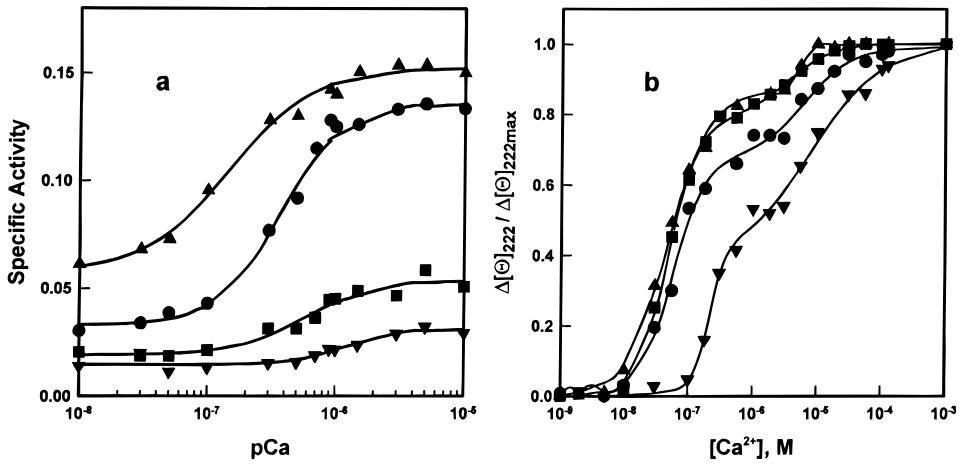

FIGURE 4: (a) Ca^{2+} dependence of the regulated actomyosin ATPase activity with wild type, D89A, αh89A, and αh89D. Conditions: The assay was carried out as described in Figure 3 except at a saturating TnC concentration (1 μM) and with 0.45 mM CaEGTA. The free Ca^{2+} concentration was controlled using CaEGTA buffer system (Sillen & Martel, 1964; Perrin & Sayce, 1967). ● wild type; ■ D89A; ▼ αh89A; ▲ αh89D. (b) Ca^{2+} titration of the change in ellipticity at 222 nm. Wild type and TnC mutants (0.1 mg/mL, in 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.9 mM EGTA, and 0.9 mM nitrilotriacetic acid, 25 °C in a 2 mM cuvette) were titrated with increasing Ca^{2+} (Dobrowolski et al., 1991b; Ramakrishnan & Hitchcock-DeGregori, 1995). The curves have been fit for two classes of sites. A representative data set is shown here. The parameters averaged from three data sets are presented in Table 3. ● wild type; ■ D89A; ▼ αh89A; ▲ αh89D.

Table 3: Calcium Binding to Troponin Cs[a]

	high affinity sites			low affinity sites		
protein	K_d (M × 10^8)	fractional change ($\Delta\theta_1$)	Hill coeff, H_1	K_d (M × 10^6)	fractional change ($\Delta\theta_2$)	Hill coeff, H_2
WT	6.8 ± 2.1	0.70 ± 0.03	2.0 ± 0.30	4.8 ± 1.4	0.30 ± 0.03	1.2 ± 0.1
αh89A	0.3 ± 0.08	0.30 ± 0.07	3.2 ± 0.46	4.1 ± 3.8	0.70 ± 0.04	0.7 ± 0.3
D89A	3.7 ± 1.5	0.79 ± 0.06	1.6 ± 0.25	4.3 ± 1.3	0.21 ± 0.06	1.6 ± 0.7
αh89D	2.5 ± 1.9	0.81 ± 0.07	1.4 ± 0.40	5.5 ± 1.1	0.19 ± 0.06	1.1 ± 0.2
pprep	7.1 ± 2.0	0.71 ± 0.05	2.5 ± 0.5	5.0 ± 1.4	0.29 ± 0.05	1.0 ± 0.75
rcrep[b]						

[a] The values given here are the parameters ± standard deviation from three data sets for each mutant as reported by SigmaPlot. A representative data set is shown in Figure 4b and Figure 5b. [b] In three independent titrations, the evidence for two transitions was not as clear as for the other TnC mutants. Therefore, we have not listed the reported parameters.

relieved TnI inhibition, but αh89A was less effective than wild type (0.50 μM vs 0.23 μM for half-maximal activity) and αh89D was slightly more effective (0.15 μM).

Ca^{2+} Dependence of TnC Regulatory Function. Figure 4a shows the Ca^{2+} dependence of the relief of TnIT inhibition of actomyosin ATPase activity by the TnCs. The Ca^{2+} concentration required for the half-maximal activation (K_d) of actomyosin ATPase activity by αh89D was lower than for wild type TnC (αh89D, $1.6 \pm 0.2 \times 10^{-7}$ M; wild type, $4.6 \pm 0.6 \times 10^{-7}$ M, $n = 4$). It was difficult to obtain actual K_d values for the Ca^{2+} dependence of the relief inhibition of actomyosin ATPase activity by D89A or αh89A because of the poor activation, though αh89A appeared to be less Ca^{2+} sensitive.

Calcium Affinity of Central Helix Mutants. We measured the Ca^{2+} binding to the low and high affinity sites of TnC indirectly using CD by taking advantage of the increase in ellipticity at 222 nm upon Ca^{2+} binding (Hinke et al., 1978; Golosinska et al., 1991). Figure 4b shows the Ca^{2+} titration curve of wild type and TnC mutants. The increase in ellipticity in D89A and αh89D when Ca^{2+} bound to the high affinity sites was greater than for wild type TnC (Table 3). When Ca^{2+} bound to the high affinity sites of αh89A, the ellipticity increased by only about 30%, compared to 70% for wild type (Figure 4b and Table 3), consistent with the effect of Mg^{2+} (Table 2). The Ca^{2+} affinity of the high affinity sites αh89D and D89A was similar to wild type, but that of αh89A was more than 10-fold lower. However, there was no significant change in the Ca^{2+} binding to low affinity sites of the TnC mutants (Table 3), despite the differences in the Ca^{2+} dependence of the ATPase (Figure 4a).

The Effect of Mutation of D89 in a Non-α-helical Background. We have shown that D89 is critical for function irrespective of the sequence of the helical environment. Two additional mutants were constructed in which D89 was mutated to Pro in a rigid polyproline linker (pprep) or to Arg in a flexible linker (rcrep, Table 1). In pprep, a six-residue polyproline linker replaced residues 87−97 of the wild type central helix. We expect this linker should form a rigid two turn, all *trans*-polyproline II-like helix (Deber et al., 1970). The length between the Ca^{2+}-binding domains may increase slightly (18.6 Å vs 16.5 Å of wild type), but this change should not affect the function (Ramakrishnan & Hitchcock-DeGregori, 1995). In rcrep, residues 87−97 were replaced with an 11- residue linker with G at every alternate residue, a sequence that should result in a flexible, random coil structure. The charges in the linker were distributed so that the linker was more positive in the N-terminal half and negative in the C-terminal half, as in the wild type sequence.

Both rcrep and pprep were defective in the activation of actomyosin ATPase activity in the presence of Ca^{2+}, having only about 15 ± 1% ($n = 4$) of the wild type maximal activity (Figure 5a), comparable to αh89A (Figure 2a). The loss of activity was much more severe than when the polyproline or random coil sequences were inserted in the

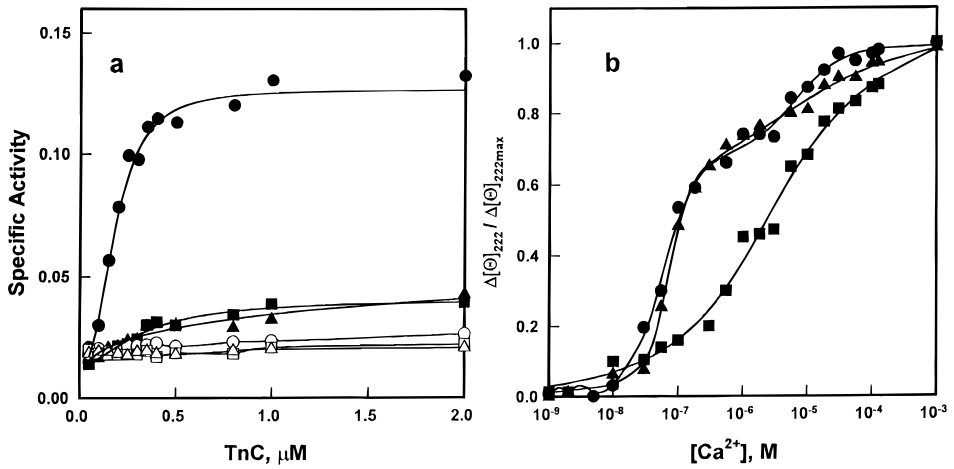

FIGURE 5: (a) Regulation of the actomyosin ATPase activity by wild type, rcrep, and pprep. Conditions: The assay was carried out as described in Figure 2a. ○, ● wild type; □, ■ rcrep; △, ▲ pprep. Filled symbols: 0.1 mM $CaCl_2$; open symbols: 0.2 mM EGTA. (b) $Ca2^+$-titration of the change in ellipticity at 222 nm. Conditions: The titration was carried out as described in Figure 4b. ● wild type; ■ rcrep; ▲ pprep.

D/E linker, where D89 was retained (Ramakrishnan & Hitchcock-DeGregori, 1995). Both mutants bound to the reconstituted thin filament in the presence and absence of Ca^{2+}, assayed by cosedimentation (results not shown). They also formed Ca^{2+}-dependent complexes with TnT (Figure 3a) and TnI (results not shown). Figure 5b shows the Ca^{2+} titration curve of the ellipticity at 222 nm of wild type, rcrep, and pprep. In pprep, the increase in ellipticity when Ca^{2+} binding to the high and low affinity sites was comparable to that of wild type (Table 3). The rcrep mutant was severely affected and showed no clear evidence for two transitions. Our results indicate that mutation of Asp89 severely impairs TnC-dependent Ca^{2+} signaling on the thin filament, irrespective of the structural environment of the mutation.

DISCUSSION

The major conclusion from the present investigation is that D89, a conserved residue in the TnC central helix, is crucial for thin filament activation in the presence of Ca^{2+}, both in the naturally occurring central helix and in a designed central helix, indicating the requirement is independent of the helical background. Moreover, mutation of D89 in a polyproline or random coil linker structure also results in failure to activate. On the basis of our results, we specifically suggest that D89 is necessary for Ca^{2+}-dependent signal transduction, not Ca^2 binding or binding to TnC targets on the thin filament. Thus, Ca^{2+} binding and signalling events can be dissociated.

As discussed in the introduction, Ca^{2+} binding to the N-terminal Ca^{2+}-specific regulatory sites results in the movement of the BC helices away from the AD helices, exposing Q51 and Q85, residues that interact with D89 in the X-ray structure (Gagné et al., 1995; Slupsky & Sykes, 1995). It is not clear how D89 relates to the exposed region in the presence of Ca^{2+}, as residues 85−94 are poorly ordered in the solution structure. Mutation of D89 to A would disrupt the salt bridge and hydrogen bonds, if they occur in physiological conditions (see Figure 1). Our results show the interactions, not present when D89 is mutated, may be critical for thin filament activation but not for determining the affinity of the Ca^{2+}-specific sites, binding to TnI or TnT, or for maintaining TnC (and the thin filament) in the off state. Our results are in agreement with those of Pollesello et al. (1994), who found that the comparable mutation in cardiac TnC did not affect Ca^{2+} affinity although it reduced the Ca^{2+}-sensitizing effect of the drug, levosimedan, which they propose binds to D89 and other hydrophobic residues more N-terminal in the D-helix. Fujimori et al. (1990) reported that an E88K mutant was only able to restore 50% of the tension restored by wild type TnC in TnC-depleted muscle fibers.

While E88 and D89 are important for function, other residues in the D-helix N-terminal to E88 that face the BC helices, and are exposed upon Ca^{2+} binding to the low affinity sites, do not appear to be critical for thin filament regulation. Mutations of residues corresponding to M82, Q85, and M86 to Cys have little effect on function (Grabarek et al., 1990; Lin et al., 1996). Introduction of bulky groups to alter the hydrophobic surface at cardiac TnC sites corresponding to residues 85 and 86 had little effect on TnC function, though modification of residue 82 resulted in lower activation (Lin et al., 1996). Similarly, mutations of K91, G92, and K93 have little effect on function (Reinach & Karlsson, 1988; Ding et al., 1994).

The TnC mutants reported here all bound to the reconstituted thin filament and showed Ca^{2+}-dependent binding to TnI and TnT on polyacrylamide gels, except αh89D which bound to TnT in the absence of Ca^{2+} as well as in its presence. All mutants fully relieved inhibition of the actomyosin ATPase by TnI, implying that the requirement for D89 is manifested in the ternary complex or on the fully-reconstituted thin filament. D89 may be crucial for Ca^{2+}-dependent TnT binding: αh89D bound to TnT in the absence of Ca^{2+} in native polyacrylamide gels. The stronger TnT binding may be responsible for the observed activation of the ATPase in the absence of Ca^{2+}.

We have previously reported that TnC in which residues ^{88}EDA^{90} were deleted was unable to activate (potentiate) the actomyosin ATPase whereas a ^{87}KEDAKGK^{93} deletion mutant had full activity in the presence of Ca^{2+} (Dobrowolski et al., 1991b). In the seven-residue deletion, E95 and E96 would be in a position to compensate for the loss of E88

and D89. This was the first report that TnC may be involved in activation (potentiation) of the actomyosin ATPase, a phenomenon known to depend on TnT (Greaser & Gergely, 1971). Other recent studies have inferred that TnC can directly interact with TnT to activate the actomyosin ATPase (Potter et al., 1995). We suggest that TnC D89, as well as E88, are critical for the activation.

ACKNOWLEDGMENT

We thank Dr. M. P. Schlunegger for the help in molecular modeling.

REFERENCES

Adler, A. J., Greenfield, N. J., & Fasman, G. D. (1973) *Methods Enzymol. 27*, 675−735.
Babu, Y. S., Bugg, C. E., & Cook, W. J. (1988) *J. Mol. Biol. 203*, 191−204.
Babu, A., Rao, V. G., Su, H., & Gulati, J. (1993) *J. Biol. Chem. 268*, 19232−19238.
Deber, C. M., Bovey, F. A., Carver, J. P., & Blout, E. R. (1970) *J. Am. Chem. Soc. 92*, 6191−6198.
Ding, X.- L., Akella, A. B., Su, H., & Gulati, J. (1994) *Protein Sci. 3*, 2089−2096.
Dobrowolski, Z., Xu, G.-Q., Chen, W., & Hitchcock-DeGregori, S. E. (1991a) *Biochemistry 30*, 7089−7096.
Dobrowolski, Z., Xu, G.-Q., & Hitchcock-DeGregori, S. E. (1991b) *J. Biol. Chem. 266*, 5703−5710.
Evans, S. V. (1993) *J. Mol. Graphics 11*, 134−138.
Farah, C. S., & Reinach, F. C. (1995) *FASEB J. 9*, 755−767.
Fujimori, K., Sorenson, M., Herzberg, O., Moult, J., & Reinach, F. C. (1990) *Nature 345*, 182−184.
Gagné, S. M., Tsuda, S., Li, M. X., Smillie, L. B., & Sykes, B. L. (1995) *Nature Struct. Biol. 2*, 784−796.
Goa, J. (1954) *Scand. J. Clin. Lab. Invest. 5*, 218−222.
Golosinska, K., Pearlstone, J. R., Borgford, T., Oikawa, K., Kay, C. M., Carpenter, M. R., & Smillie, L. B. (1991) *J. Biol. Chem. 266*, 15797−15809.
Grabarek, Z., Tan, R.-Y., Wang, J., Tao, T., & Gergely, J. (1990) *Nature 345*, 132−135.
Grabarek, Z., Tao. T., & Gergely, J. (1992) *J. Muscle Res. Cell Motil. 13*, 383−393.
Greaser, M. L., & Gergely, J. (1971) *J. Biol. Chem. 246*, 4226−4233.
Greenfield, N. J., & Fasman, G. D. (1969) *Biochemistry 8*, 4108−4116.
Gulati, J., Babu, A., Su, H., & Zhang, Y. F. (1993) *J. Biol. Chem. 268*, 11685−11690.
Head, J. F., & Perry, S. V. (1974) *Biochem. J. 137*, 145−154.
Herzberg, O., & James, M. N. G. (1988) *J. Mol. Biol. 203*, 761−779.
Herzberg, O., Moult, J., & James, M. N. G. (1986) *J. Biol. Chem. 261*, 2638−2644.
Hinke, M. T., McCubbin, W. D., & Kay, C. M. (1978) *Can. J. Biochem. 56*, 384−395.
Hitchcock-DeGregori, S. E., Mandala, S., & Sachs, G. A. (1982) *J. Biol. Chem. 257*, 12573−12580.
Hitchcock-DeGregori, S. E., Lewis, S. F., & Chou, T. M.-T. (1985) *Biochemistry 24*, 3305−3314.
Johnson, J. D., & Potter, J. D. (1978) *J. Biol. Chem. 253*, 3775−3777.
Kawasaki, Y., & van Eerd, J. P. (1972) *Biochem. Biophys. Res. Commun. 49*, 898−905.
Kraulis, P (1991) *J. Appl. Crystallogr. 24*, 946−950.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lin, X., Dotson, D. G., & Putkey, J. A. (1996) *J. Biol. Chem. 271*, 244−249.
Margossian, S. S., & Lowey, S. (1982) *Methods Enzymol. 85*, 55−71.
Marqusee, S., & Baldwin, R. L. (1987) *Proc. Natl. Acad. Sci. U.S.A 84*, 8898.
Perrin, D. D., & Sayce, J. G. (1967) *Talanta 14*, 833−842.
Pollesello, P., Ovaska, M., Kaivola, J., Tilgmann, C., Lundström, K., Kalkkinen, N., Ulmanen, I., Nissinen, E., & Taskinen, J. (1994) *J. Biol. Chem. 269*, 28584−28590.
Potter, J. D. (1982) *Methods Enzymol. 85*, 241−263.
Potter, J. D., Sheng, J., Pan, B-S., & Zhao, J. (1995) *J. Biol. Chem. 270*, 2557−2562.
Ramakrishnan, S., & Hitchcock-DeGregori, S. E. (1995) *Biochemistry 34*, 16789−16796.
Ramakrishnan, S., & Hitchcock-DeGregori, S. E. (1996) *Biophys. J. 70*, A25.
Reinach, F. C., & Karlsson, R. (1988) *J. Biol. Chem. 263*, 2371−2376.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Satyshur, K. A., Rao, S. T., Pyzalska, D., Drendel, W., Greaser, M., & Sundaralingam, M. (1988) *J. Biol. Chem. 263*, 1628−1647.
Sillen, L. G., & Martel, A. E. (1964) in *Stability Constants of Metal Complexes*, 2nd ed., Special Publication No. 17, The Chemical Society, Burlington House, London.
Slupsky, C. M., & Sykes, B. D. (1995) *Biochemistry 34*, 15953−15964.
Smith, L., Greenfield, N. J., & Hitchcock-DeGregori, S. E. (1994) *J. Biol. Chem. 269*, 9857−9863.
Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60−93.
Tobacman, L. S. (1996) *Annu. Rev. Physiol. 58*, 447−481.
White, H. D. (1982) *Methods Enzymol. 85*, 698−708.
Xu, G.-Q., & Hitchcock-DeGregori, S. E. (1988) *J. Biol. Chem. 263*, 13962−13969.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468−500.

BI961788U