Analysis of the Regulatory and Structural Defects of Troponin C Central Helix Mutants†

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Received February 1, 1991; Revised Manuscript Received April 15, 1991

ABSTRACT: Five deletion mutants of the D/E linker region of the troponin C central helix were tested for conformational and functional differences from wild-type troponin C. The mutants were in the region $_{87}$KEDAKGKSEEE$_{97}$: dEDA, dKG, dKGK, dKEDAKGK, and dSEEE, designed to change the length of the central helix and the orientation of the Ca^{2+}-binding domains relative to each other [Dobrowolski, Z., Xu, G.-Q., & Hitchcock-DeGregori, S. E. (1991) *J. Biol. Chem. 266*, 5703–5710]. Previous work showed that all mutants except dSEEE are partially defective in one part of the Ca^{2+} switch or the other. All mutants undergo Ca^{2+}-dependent conformational changes as detected by changes in electrophoretic mobility, α-helix content, and hydrophobic exposure. Deletions of the central helix do not extensively alter the thermal stability of troponin C, as determined by temperature-dependent loss of α-helix. There are differences among the mutants that do not correlate with function. All troponin C mutants show Ca^{2+}-dependent interaction with troponin I and T in polyacrylamide gels. Troponin I–troponin C interaction was also analyzed by Ca^{2+}-dependent increase in the monomer/excimer ratio of troponin I and relief of inhibition of the actomyosin S1 ATPase. While all mutants retain basic function, dKGK, dKEDAKGK, and dEDA have altered interaction with troponin I in the absence of Ca^{2+}. dSEEE differs in conformation from wild type, but it is normal in functional assays. This conserved region of the D/E linker is not required for interaction with troponin I in the presence or absence of urea.

In striated muscles, troponin C, together with troponin I and troponin T and tropomyosin, regulates contraction in a Ca^{2+}-dependent manner [Hartshorne & Mueller, 1968; Greaser & Gergely, 1971; reviewed in Leavis and Gergely (1984); Zot & Potter, 1987]. Calcium binding to troponin C activates the thin filament.

The four Ca^{2+}-binding sites in troponin C are found in two globular domains connected to each other by an unusual long α-helix that is fully exposed in the middle, the "D/E linker" (Herzberg & James, 1988; Satyshur et al., 1988). The two sites in the carboxyl-terminal domain bind Mg^{2+} or Ca^{2+} with high affinity and in physiological conditions are occupied by divalent cations at all times (Potter & Gergely, 1975). The amino-terminal Ca^{2+}-specific sites are referred to as the regulatory sites because occupancy by Ca^{2+} is required for activation of contraction (Potter & Johnson, 1982).

Calcium or Mg^{2+} binding to the high-affinity sites results in a major conformational change illustrated by a large increase in the α-helix, probably restricted to the carboxyl-terminal domain (Kawasaki & van Eerd, 1972; McCubbin & Kay, 1973; Leavis et al., 1978; Johnson & Potter, 1978). In addition, the exposure of hydrophobic groups in troponin C and calmodulin increases upon Ca^{2+} binding. Troponin I competes with the hydrophobic probes for troponin C, presumably by binding to the same regions (LaPorte et al., 1980; Tanaka & Hidaka, 1980, 1981; Golapakrishna & Anderson, 1982; Brzeska et al., 1983a; Tanaka et al., 1984).

The exposed region of the central helix, especially residues 89–100 [rabbit sequence (Collins et al., 1977), corresponding to residues 92–103 in the chicken sequence (Wilkinson, 1976)] is postulated to be crucial for binding to troponin I and T and for Ca^{2+}-dependent regulation (Leavis et al., 1978; Weeks & Perry, 1978; Grabarek et al., 1981; Hitchcock, 1981; Dobrovol'sky et al., 1984; Leszyk et al., 1987, 1988). Another suggested role for the central helix is interdomain transmission of information concerning occupancy of the Ca^{2+}-binding sites (Johnson et al., 1978, 1979; Iio & Kondo, 1981; Potter et al., 1976; Wang et al., 1982; Kareva et al., 1986; Cheung et al., 1982; Grabarek et al., 1986; Wang & Gergely, 1986).

We made a series of deletion mutants to investigate the function of the troponin C central helix. They are in the exposed D/E linker region $_{87}$KEDAKGKSEEE$_{97}$ in the avian sequence (Wilkinson, 1976): dEDA, dKG, dKGK, dSEEE, and dKEDAKGK. The mutations would decrease the length of the central helix from 3.0 to 10.5 Å (if the D/E linker is helical), bringing the two Ca^{2+}-binding domains closer to each other. Also, the deletions would theoretically change the orientation of the Ca^{2+}-binding domains relative to each other. In previously reported work, we analyzed the basic functional properties of the five mutants (Dobrowolski et al., 1991). We found that dSEEE functioned normally in all assays. The other four were partially defective in one part of the Ca^{2+} regulatory switch or the other. dEDA and dKG were unable to fully activate the actomyosin S1 ATPase in the presence of Ca^{2+}. dEDA did not activate above the level of actomyosin S1 alone in the presence of Ca^{2+}. In contrast, dKGK and dEDAKGK only partially maintained the system in the in-

† This research was supported by grants from the National Institutes of Health (GM36326) and the Muscular Dystrophy Association to S. E.H.-D. and by postdoctoral fellowships from the American Heart Association, New Jersey Affiliate, to Z.D. and G.-Q. X.

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0006-2960/91/0430-7089$02.50/0

hibited state in the absence of Ca^{2+}, though they functioned normally in the presence of Ca^{2+}. They all bound Ca^{2+} to the low- and high-affinity sites. In troponin C depleted skinned fibers, only dKG was impaired and required higher protein and Ca^{2+} concentrations to restore force development (Sheng et al., 1991). The results show that changes in the central helix can alter the regulatory function of troponin C.

In the present work we have carried out further analysis of these mutants in order to understand at the molecular level the reason for their dysfunction. Portions of this work have been published in a preliminary form (Dobrowolski et al., 1989; Dobrowolski & Hitchcock-DeGregori, 1990).

Materials and Methods

Mutant Construction, Expression, and Protein Purification. Five troponin C deletion mutants were made with use of oligonucleotide-directed mutagenesis as previously described (Xu & Hitchcock-DeGregori, 1988; Dobrowolski et al., 1991). The mutations were all deletions of the D/E linker, $_{87}$KEDAKGKSEEE$_{97}$ (Figure 1). The mutant cDNAs were cloned in pUC120 and expressed in *Escherichia coli* strain PAM 163, in some instances carrying a plasmid that expresses lac repressor.

The recombinant troponin Cs were purified from bacterial lysates with use of ammonium sulfate fractionation, chromatography on phenyl-Sepharose CL4B or DE52 cellulose and finally (for most experiments) by HPLC reverse-phase chromatography on a Bio-Rad RP304 column (Dobrowolski et al., 1991). The purity of the protein was evaluated by SDS and urea/polyacrylamide gel electrophoresis and by UV absorption spectroscopy.

Circular Dichroism Spectroscopy. The far-ultraviolet circular dichroism spectra (190–280 nm) of recombinant troponin Cs were determined on an Aviv 60a circular dichroism spectrophotometer. The proteins were stored frozen (−20 °C) at 2 mg/mL. The night before the measurements were to be made, the proteins were diluted to 0.4 mg/mL in the buffer containing the appropriate divalent cation. (See legend to Figure 3.) The spectra shown are the averages of two scans. The mean residue ellipticity (θ) was calculated according to Adler et al. (1973) and the percent α-helix according to Greenfield and Fasman (1969) was calculated from

$$\% \ \alpha\text{-helix} = \frac{[\theta]_{208nm} - 4000}{33\,000 - 4000} \qquad (1)$$

The temperature dependence of the ellipticity at 222 nm was determined by increasing the temperature from 15 to 90 °C at a rate of 0.66 deg/min with use of a Perkin-Elmer electronic controller. The far-UV circular dichroism spectrum was taken before thermal denaturation, at 90 °C, and after renaturation to 15 °C. The initial and final spectra were indistinguishable.

Analytical HPLC Chromatography. Hydrophobic interaction chromatography was carried out on a Bio-Gel TSK phenyl-5PW column (7.5 × 75 mm) at room temperature. To vary the occupancy of the troponin C Ca^{2+}-binding sites, we employed three different buffers: (1) 50 mM Tris-HCl, pH 7.4/0.01 mM EDTA;[1] (2) 50 mM Tris-HCl, pH 7.4; and (3) 50 mM Tris-HCl, pH 7.4/5 mM $CaCl_2$. These buffers were used to prepare eluants for the HPLC. Buffer A contained one of the three buffers plus 0.75 M $(NH_4)_2SO_4$ (Schwarz/Mann Biotech, less than 0.1 ppm of calcium impurity); buffer B contained one of the three buffers with 50% acetonitrile (HPLC grade).

For each cycle, about 15 μg of troponin C was injected into a column equilibrated in 100% A, followed by a 10-min linear gradient from 100% A to 50% B and 2 min at 50% B before recycling. The flow rate was 1 mL/min. The void eluted at 2.6 min. Peaks were detected at 260 nm. The peak elution time was reported by the Hitachi HPLC system. The identity of the troponin C peak was verified by SDS/polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gels (with or without 6 M urea) for analysis of complex formation of troponin C with troponin I were prepared and run according to Head and Perry (1974). Nondenaturing polyacrylamide gel electrophoresis for analysis of binding to troponin T was carried out according to the method of Head and Perry (1974) with two modifications: the pH of the gel was increased to 9.1 and glycerol was omitted from the gel. SDS/polyacrylamide gels were run according to Laemmli (1970).

ATPase Measurements. Troponin I was dialyzed against 0.5 M NaCl/10 mM imidazole, pH 7.0/0.5 mM dithiothreitol. Variable amounts of troponin C were added to reconstituted thin filaments containing troponin I, tropomyosin, and actin.

ATPase assays were carried out in microtiter plates floated in a water bath or in a thermoequilibrated Molecular Devices ThermoMax microtiter plate reader as previously described (Dobrowolski et al., 1991). The assays were single-point determinations. Time courses were carried out to show that phosphate liberation was linear over the times of the experiments.

Fluorescence Measurements. The labeling of troponin I with *N*-(1-pyrenyl)maleimide (Molecular Probes, Inc.) was carried out as described by Strasburg et al. (1985). Since chicken troponin I lacks the Cys 133 found in rabbit troponin I (Wilkinson & Grand, 1978), it was not necessary to block this residue in intact troponin prior to labeling. The degree of labeling was about 0.9 mol of pyrene/mol of cysteine.

The steady-state fluorescence measurements were made with use of a Perkin-Elmer 650-10S fluorescence spectrophotometer. The experiments were carried out at 10 °C in 50 mM PIPES, pH 7.0/100 mM NaCl/0.45 mM EGTA. To measure the Ca^{2+} dependence of the fluorescence response, increasing amounts of 4.5 mM $CaCl_2$, containing 5 mM NaOH to maintain a constant pH during titration, were added. The free Ca^{2+} concentrations with the EGTA buffer system were determined from titration curves (gift of Dr. Z. Grabarek) based on binding constants published by Sillen and Martel (1967), calculated according to Perrin and Sayce (1967). Corrections were made for dilution and Raman light scattering, if necessary. The initial volume was 1 mL.

The experimental data were analyzed on an IBM-AT computer with use of PENNZYME, a curve fitting and statistical analysis program (Kohn et al., 1979). The experimental data were fit to the equation

$$F_{(i)} = F_0 + dF_{max}\frac{[Ca^{2+}]^H_{(i)}K_a^H}{1 + [Ca^{2+}]^H_{(i)}K_a^H} \qquad (2)$$

for the calcium-dependent changes in fluorescence (monomer/excimer fluorescence ratio) of pyrene–troponin I–troponin C complexes, where $F_{(i)}$ = fluorscence signal after i^{th} addition of Ca^{2+}, $[Ca^{2+}]_{(i)}$ = free calcium ion concentration after the i^{th} addition, F_0 and dF_{max} = initial fluorescence signal and maximal signal increase, respectively, K_a = association con-

[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; NTA, nitrilotriacetic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TM, tropomyosin; TnC, troponin C; TnI, troponin I; TnT, troponin T.

Wildtype	$_{87}$K E D A K G K S E E E$_{97}$
dEDA	K - - - K G K S E E E
dKG	K E D A - - K S E E E
dKGK	K E D A - - - S E E E
dSEEE	K E D A K G K - - - -
dKEDAKGK	- - - - - - - S E E E

FIGURE 1: Deletions of the troponin C central helix.

stant, and H = Hill coefficient. Three sets of data (obtained using the same pyrene–troponin I preparation) were combined prior to curve fitting:

$$F_{(i)} = F_0 + dF_{max}\{Y + X_{(i)} + 1/K_a - [(Y + X_{(i)} + 1/K_a)^2 - 4YX_{(i)}]^{1/2}\}/2Y \quad (3)$$

for titration of pyrene–troponin I by troponin C in the presence of calcium where $F_{(i)}$ = fluorescence signal after i^{th} addition of troponin C, F_0 and dF_{max} = initial fluorescence signal and maximal increase of signal, respectively, $X_{(i)}$ = concentration of the variable component (troponin C) after i^{th} addition, Y = concentration of the invariable component (pyrene–troponin I, corrected for dilution), and K_a = complex association constant. Each data set was fitted separately due to the scatter of dF_{max} values between experiments. The equation is based on an equation given by Ingraham and Swenson (1984).

General Methods. Contractile proteins, other than the recombinant troponin Cs, were prepared with use of the following previously published methods: rabbit skeletal actin (Hitchcock-DeGregori et al., 1982), chicken pectoral muscle myosin papain S1 (Margossian & Lowey, 1982) (gift of Dr. D. Winkelmann), troponin I from chicken pectoral muscle (for the labeling and ATPases) and troponin I and T from rabbit back and leg muscle (for the electrophoretic analyses) (Hitchcock et al., 1981), and chicken α-tropomyosin (Hitchcock-DeGregori et al., 1985).

Protein concentrations were determined with the use of a biuret assay with bovine serum albumin as a standard (Itzhaki & Gill, 1964) or with spectrophotometric methods and the following extinction coefficients ($A_{280}^{1\%}$): actin, 11.0; myosin S1, 8.3; and tropomyosin, 3.0. For some experiments the troponin C concentration was determined with the BCA assay (Pierce Chemical Co.). With the BCA assay, a correction was made for the difference in troponin C and bovine serum albumin after standardization with the biuret assay. Troponin C has no tyrosine or tryptophan. Determination of the protein concentration based on phenylalanine absorbance at 259 nm is risky except for highly purified protein due to possible contamination by nucleic acid.

All chemicals were reagent grade. The water used was house-deionized, passed through an organic removal cartridge and a high-purity ion-exchange resin, and finally glass distilled.

Results

In order to determine the molecular basis for the functional differences we have observed in our troponin C central helix deletion mutants (Figure 1), we analyzed the conformation and interaction with troponin I and T. Our major conclusions follow. (1) All mutants undergo Ca^{2+}-dependent conformational changes. However, there are structural differences among the troponin Cs that cannot be correlated with function. (2) All troponin C mutants show Ca^{2+}-dependent interaction with troponin I and T. The regulatory dysfunction of dEDA, dKGK, and dKEDAKGK is due at least in part to altered interaction with troponin I in the absence of Ca^{2+}. (3) The conserved charge cluster, SEEE, is not required for Ca^{2+}-dependent troponin I binding in the presence of 6 M urea, in contrast to studies with troponin C peptides (Leavis et al., 1978; Grabarek et al., 1981).

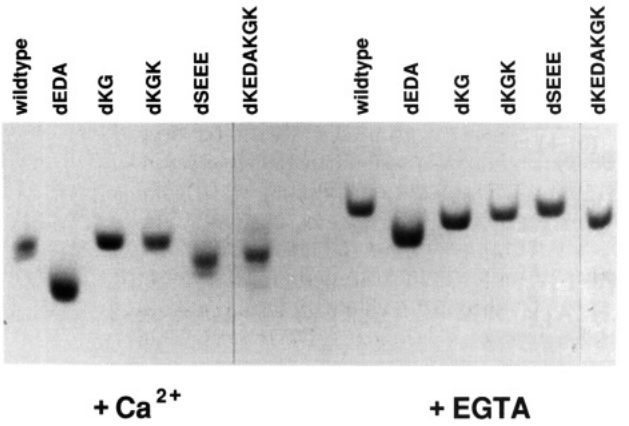

FIGURE 2: Calcium-dependent migration of troponin Cs in urea/polyacrylamide gels. Electrophoresis of troponin C in 8% polyacrylamide gels containing 6 M urea (Head & Perry, 1974). The troponin C samples were applied in sample buffer containing 5 mM $CaCl_2$ or 5 mM EGTA. The doublet bands in the presence of calcium are due to incomplete saturation with calcium not impurities.

Conformational Analysis of Free Troponin Cs

All Mutants Show Ca^{2+}-Dependent Migration in Urea/Polyacrylamide Gels. Figure 2 shows that all five mutants, like wild-type troponin C, migrated faster in the presence of Ca^{2+}. The mobilities of the mutants differed, seen best in the absence of Ca^{2+} in urea/polyacrylamide gels. The differences must be primarily due to conformation rather than charge since the mobilities of dKGK and dSEEE were similar to each other and to wild type even though they differ in net charge by 5. The change in mobility upon Ca^{2+} binding differed among mutants; that of dKGK was smaller than wild type, and that of dSEEE was greater.

All Mutants Have Similar α-Helix Content and Show Conformational Changes upon Divalent Cation Binding to the Low- and High-Affinity Sites. The α-helix content of wild-type troponin C, calculated from the mean residue ellipticity at 208 nm, was similar to that reported for troponin C isolated from muscle, ~50% in the presence of Ca^{2+} and ~30% in the presence of EDTA (Kawasaki & van Eerd, 1972; Murray & Kay, 1972; Gruda et al., 1973; Johnson & Potter, 1978). The helicity of two mutants (dKGK and dKEDAKGK) was lower than that of wild type while dSEEE had higher helicity (Figure 3), even though one turn of the α-helix was deleted. We do not know the level of significance of these differences because it has been difficult to obtain a highly accurate measure of protein concentration. (See Materials and Methods.)

Binding of Mg^{2+} to the high-affinity sites of troponin C causes a large increase in α-helix with a slight further increase upon occupancy of all four sites with Ca^{2+} (Kawasaki & van Eerd, 1972; McCubbin & Kay, 1973; Johnson & Potter, 1978). Recombinant wild-type and mutant troponin Cs all showed this effect (Figure 3). The increases in ellipticity at 222 nm upon Mg^{2+} binding were 44–54% and 47–61% in the presence of Ca^{2+}, relative to the apotroponin Cs (with 2 mM EDTA), comparable to published values for rabbit troponin C (Kawasaki & van Eerd, 1972; Johnson & Potter, 1978). The smallest increase was in dSEEE, the mutant that had the highest α-helix content in EDTA. Altogether, the results

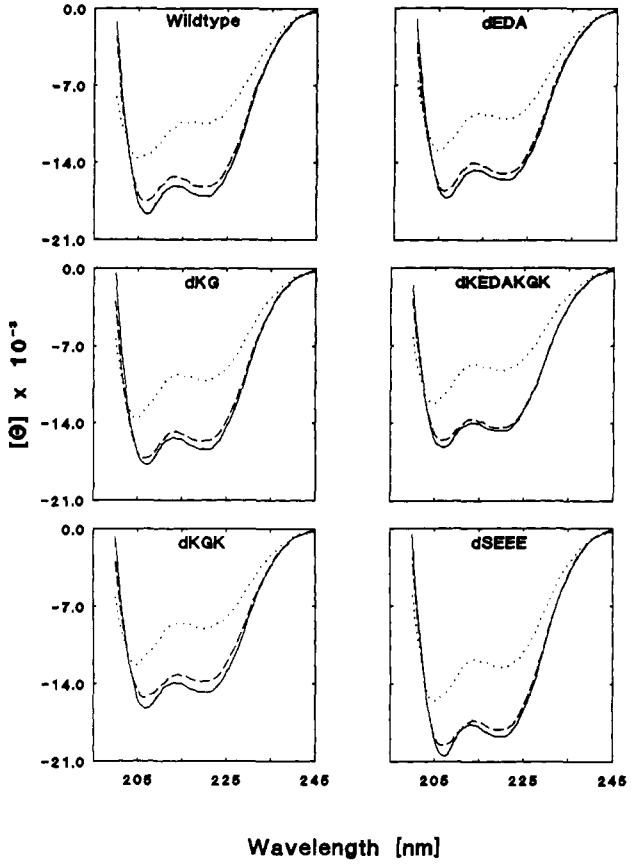

FIGURE 3: Circular dichroism spectroscopy of wild-type and mutant troponin Cs. The far-UV circular dichroism spectra were determined at 15 °C in a 1-mm cuvette as described in the Materials and Methods section. The buffer was 50 mM KCl/2 mM HEPES, pH 7.0/0.5 mM dithiothreitol containing (···) 2 mM EDTA, (- - -) 5 mM $MgCl_2$, or (—) 3 mM $CaCl_2$.

indicate that the mutations in the central helix do not dramatically alter the global secondary structure of the protein and its response to divalent cation binding.

Hydrophobicity. Calcium-dependent changes in hydrophobicity were assayed by use of quantitative hydrophobic interaction chromatography. The retention time of troponin C with an acetonitrile gradient, determined in conditions in which there would be different occupancy of the Ca^{2+} sites, was used as a measure of hydrophobicity (Figure 4). In the presence of 10 mM EDTA (apotroponin C), two mutants, dKGK and dSEEE, eluted at later times than that of wild type, indicating greater hydrophobicity (Figure 4a). In the absence of added Ca^{2+}, when the high-affinity Ca^{2+}/Mg^{2+} sites should be occupied by Ca^{2+} contaminating the solutions, all troponin Cs except for dSEEE were less hydrophobic than apotroponin C (Figure 4b). dSEEE eluted 2 min later than the others and later than apo-dSEEE. dKG eluted slightly earlier than wild type. In the presence of 5 mM $CaCl_2$, the retention time increased for all troponin Cs (Figure 4c). As in the other conditions, dSEEE eluted later than wild type, by about a minute; dKG and dKEDAKGK eluted slightly earlier. Clearly, dSEEE has the greatest hydrophobic exposure and the hydrophobicity does not decrease upon Ca^{2+} binding to the high-affinity sites. Interestingly, the regulatory function of this mutant is indistinguishable from wild type (Dobrowolski et al., 1991).

Thermal Stability. Sundaralingam et al. (1985) postulated that salt bridges between side chains in the exposed region of the central helix contribute to its stability. In three mutants (dKGK, dKEDAKGK, dSEEE), the Lys 93–Glu 97 salt

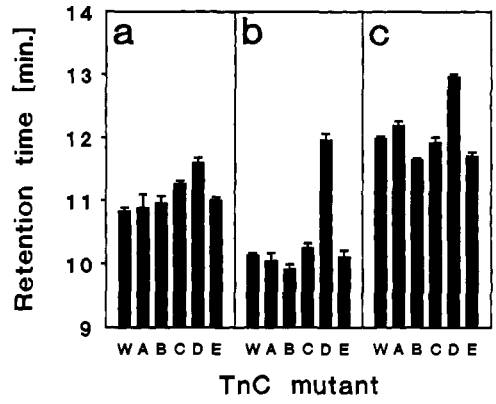

FIGURE 4: Hydrophobicity of wild-type and mutant troponin Cs. The elution time of the troponin Cs from a Bio-Gel TSK phenyl-5PW reverse-phase analytical HPLC column was measured. For details see Materials and Methods. (a) In the presence of 0.01 mM EDTA; (b) without calcium or EDTA added; (c) in the presence of 5 mM $CaCl_2$. Symbols: W, wild type; A, dEDA; B, dKG; C, dKGK; D, dSEEE; E, dKEDAKGK. The elution times, averaged from 3 to 5 repetitions, are shown with standard deviations. Since the elution time of the void was 2.6 min, all elution times were during the period of the gradient except for dSEEE in the presence of $CaCl_2$, which eluted slightly after the end of the gradient. (See Materials and Methods.)

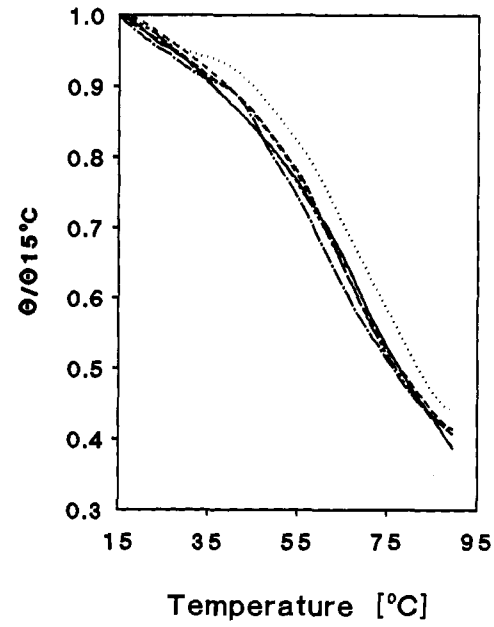

FIGURE 5: Thermal stability of wild-type and mutant troponin C. The ellipticity was measured as a function of temperature between 15 and 90 °C at 222 nm in a 1-mm cuvette. The temperature was increased at a rate of 0.66 deg/min. The troponin Cs were 0.4 mg/mL in 50 mM KCl/2 mM HEPES, pH 7.0/5 mM $MgCl_2$/1 mM EGTA/0.5 mM dithiothreitol. Symbols: (—) wild type, (···) dEDA, (- - -) dKG, (-·-) dKGK, (– –) dSEEE, (·–·) dKEDAKGK.

bridge (Herzberg & James, 1988) would be unable to form. On the other hand, Gly 92, a residue unfavorable for helix formation, has been deleted in three mutants. We measured the ellipticity at 222 nm as a function of temperature to learn if the deletions altered stability. Figure 5 shows that the main transitions of the mutant troponin Cs were similar to that of wild type, whose midpoint was 68 °C, close to that previously reported for rabbit skeletal troponin C in similar ionic conditions in which the high-affinity Ca^{2+} sites would be occupied by Mg^{2+} (72 °C; Brzeska et al., 1983b). dKEDAKGK was about 2.5 °C lower, and dEDA was about 4 °C higher. The curves had multiple transitions in some cases, and the transitions were broad. At 90 °C about 40% of the original α-helix

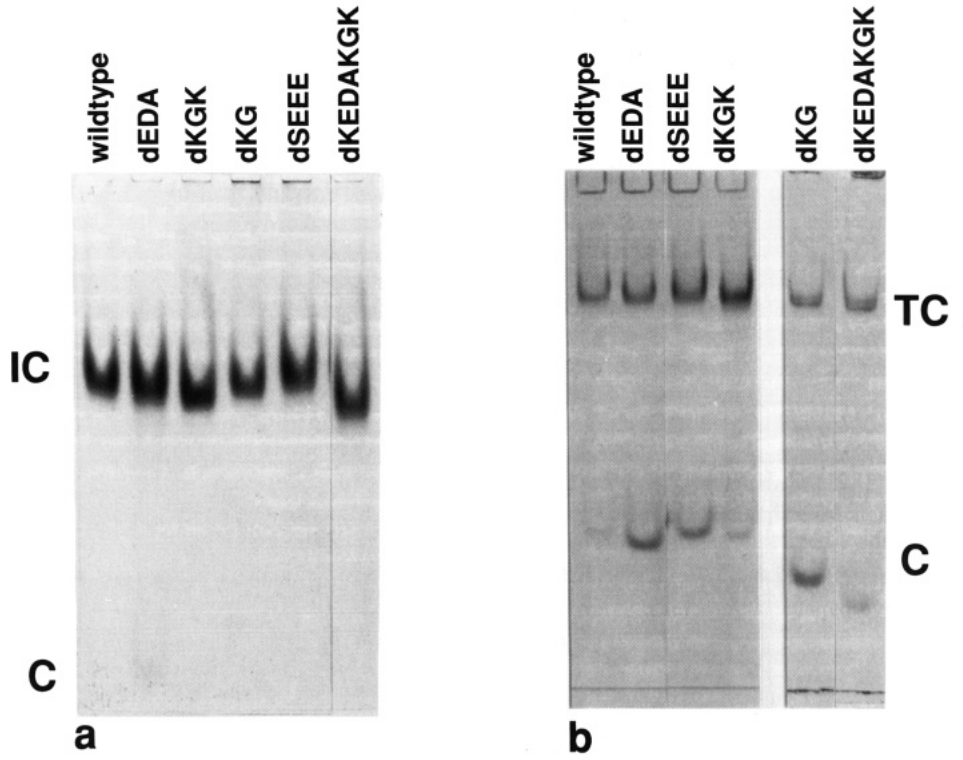

FIGURE 6: Electrophoresis of troponin I–C and troponin T–C complexes in polyacrylamide gels. (a) Troponin I and troponin C were combined in 2.4 M urea/10 mM Tris-HCl, pH 7.4/0.1 mM $CaCl_2$/6 mM dithiothreitol and analyzed on an 8% polyacrylamide gel. The troponin I–C complex migrated as a single band, unbound troponin C (if present) migrated near the front of the gel, and unbound troponin I did not enter the gel. The results were the same in gels made with or without 6 M urea. (b) Troponin T was combined with excess troponin C in 100 mM NaCl/12 mM imidazole, pH 7.0/15 mM NH_4HCO_3/0.5 mM DTT and analyzed on an 8% polyacrylamide gel. The troponin T–C complex migrated as a single band, unbound troponin C migrated near the front of the gel, and unbound troponin T did not enter the gel.

remained. We conclude that the D/E helical linker does not contribute in a major way to the overall stability of troponin C and that its structure does not affect the stability of the Ca^{2+}-binding domains.

Interaction with Troponin I and T

In the troponin complex, troponin C interacts with both troponin I and T, though the association is stronger with troponin I (Hitchcock, 1975a,b, 1981; Hitchcock & Lutter, 1975; Ingraham & Swenson, 1984; Cheung et al., 1987). We investigated the binding of the troponin C mutants to troponin I and T to learn if the impaired regulatory function is due to altered subunit interactions.

Calcium-Dependent Binding to Troponin I and T. Troponin I–troponin C complexes formed in urea can be detected in polyacrylamide gels (made with or without 6 M urea) in the presence of Ca^{2+} but not in its absence (Head & Perry, 1974). Figure 6a shows that all mutants formed troponin I–C complexes in the presence of Ca^{2+}. In the absence of Ca^{2+}, no complex formed. dSEEE lacks residues in the residue 92–103 region (residues 89–100 in rabbit) implicated to be required for binding to troponin I in the presence of urea (Leavis et al., 1978; Grabarek et al., 1981). Likewise, troponin C–troponin T complexes formed with all mutants, indicating that the affinity is not grossly reduced (Figure 6b).

Relief of Troponin I Inhibition of the Actomyosin S1 ATPase. Troponin C relieves the inhibition of the actomyosin ATPase by troponin I in the presence and absence of Ca^{2+} (Greaser & Gergely, 1971). In addition to being a functional assay, this is the best method for assaying interaction of troponin C with troponin I when only the high-affinity sites are occupied. Figure 7 shows that all mutants retained this function in an actomyosin S1 system. In the presence of Ca^{2+} (Figure 7a), the mutant troponin Cs were nearly indistinguishable from wild type, including dKG, which was less effective in the activation of fully reconstituted systems, both in vitro and in skinned fibers (Dobrowolski et al., 1991; Sheng et al., 1991).

In the absence of Ca^{2+} (Figure 7b), relief of inhibition of dEDA was less than wild type, consistent with previous results with troponin I and T (Dobrowski et al., 1991). dKGK and dKEDAKGK allowed greater relief of inhibition than wild type as we have found in actomyosin and actomyosin S1 ATPases with tropomyosin–troponin I–T in the absence of Ca^{2+} (Xu & Hitchcock-DeGregori, 1988; Dobrowolski et al., 1991). dKG and dSEEE were indistinguishable from wild type.

Interaction with Pyrene–Troponin I. When troponin C binds to rabbit troponin I labeled at Cys 48 and 64 with pyrenylmaleimide, there is an increase in the monomer/excimer ratio upon Ca^{2+} binding to the low-affinity sites of troponin C, indicative of an increase in distance between the two cysteines (Strasburg et al., 1985). We have used this assay to compare the Ca^{2+} dependence of troponin I–C interaction and the structural changes that take place in troponin I upon binding to mutant troponin Cs.

All troponin Cs showed a calcium-dependent increase in the monomer/excimer ratio of pyrene–troponin I associated with Ca^{2+} or Mg^{2+} binding to the low-affinity sites (Figure 8). Troponin C also caused a small but detectable increase in the monomer/excimer ratio in the absence of Ca^{2+} (with or without Mg^{2+}), indicative of complex formation at the protein concentrations used in the assays. The Ca^{2+} dependence of the structural change in the troponin I–C complexes was similar for all troponin Cs, with K_ds of about 1×10^{-6} M.

However, there were differences among the mutants. dKGK and dKEDAKGK caused large increases in the troponin I monomer/excimer ratio relative to wild type at low Ca^{2+}

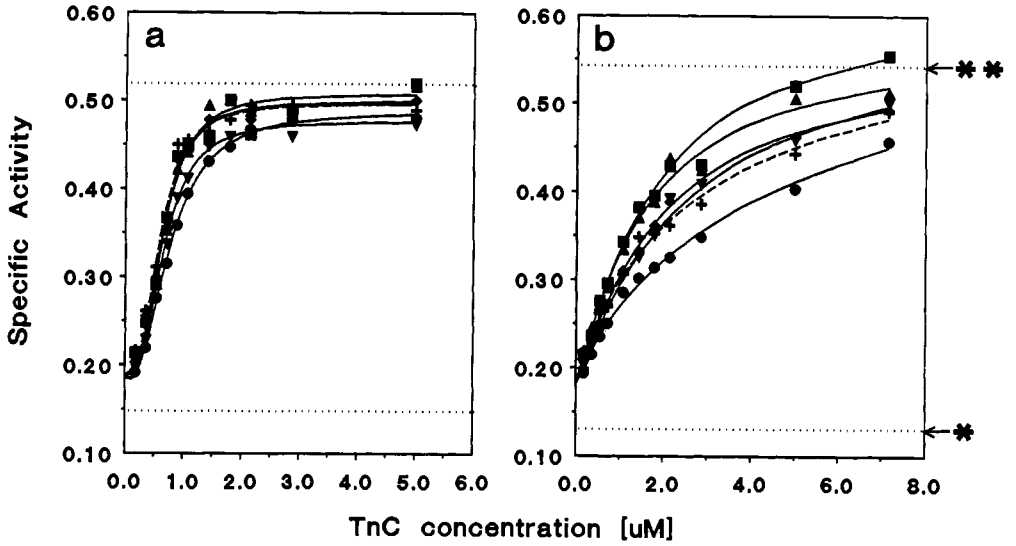

FIGURE 7: Relief of troponin I inhibition of the actomyosin S1 ATPase by wild-type and mutant troponin Cs: (a) in the presence of calcium and (b) in the absence of calcium. Conditions: 3.6 μM actin, 0.7 μM tropomyosin, 1.4 μM troponin I, 0.7 μM myosin S1, variable concentration of troponin Cs, in 25 mM NaCl/10 mM imidazole, pH 7.0/0.5 mM $MgCl_2$/4 mM MgATP/0.1 mM $CaCl_2$ or 0.2 mM EGTA, 25 °C, 30-min incubation time. The specific activity is expressed as nmol of $P_i \cdot min^{-1} \cdot$nmol of $S1^{-1}$. Symbols: + wild type (dashed line); ●, dEDA; ♦, dKG; ■, dKGK; ▼, dSEEE; ▲, dKEDAKGK; **, activity of actomyosin S1–tropomyosin (no troponin I); *, activity of myosin S1 alone. The activity of actomyosin S1 was 0.55 nmol of $P_i \cdot min^{-1} \cdot$nmol of $S1^{-1}$ in presence of Ca^{2+} and 0.62 in presence of EGTA. A representative data set is shown.

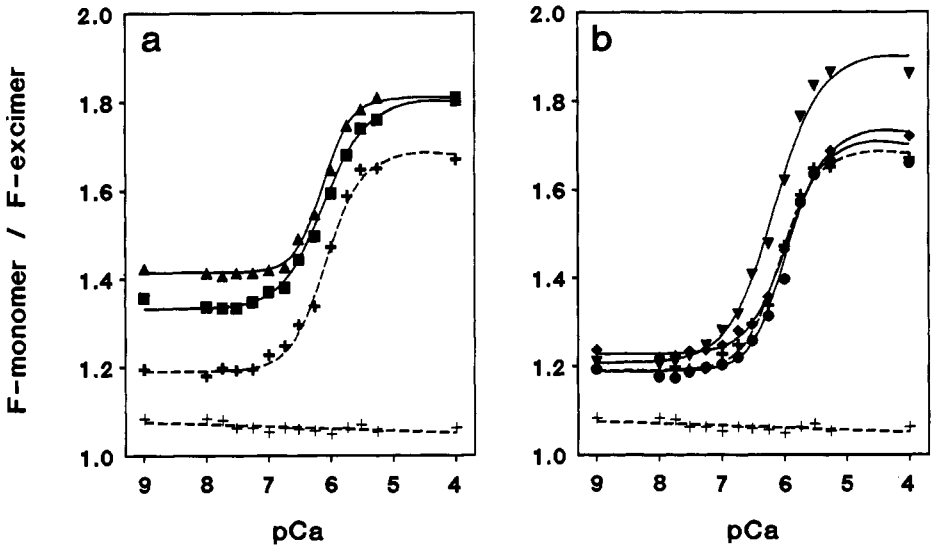

FIGURE 8: Calcium dependence of fluorescence of pyrene–troponin I–troponin C complexes: (a) wild type, dKGK, and dKEDAKGK; (b) wild type, dEDA, dKG, and dSEEE. Conditions: 0.1 μM pyrene–troponin I, 0.5 μM troponin, 50 mM PIPES, pH 7.0, 100 mM NaCl, 2 mM $MgCl_2$, 0.45 mM EGTA calcium buffer system, 10 °C, λ(Ex) = 345 nm, slit = 2.5 mm; λ(Em) = 388 nm (monomer), 485 nm (excimer), slit = 10 mm. The data points are the averaged values from three repetitions. Comparable results were obtained in the absence of $MgCl_2$. For details of the procedure and data analysis see Materials and Methods. Symbols: +, pyrene–troponin I alone (dashed line); +, wild type (dashed line); ●, dEDA; ♦, dKG; ■, dKGK; ▼, dSEEE; ▲, dKEDAKGK.

concentrations (1.33 ± <0.01 and 1.41 ± <0.01 vs 1.19 ± 0.01). The overall increase in the ratio upon Ca^{2+} binding to the low-affinity sites of dKGK (0.47 ± <0.01) was similar to that of wild type (0.49 ± 0.02); that of dKEDAKGK was slightly less (0.39 ± 0.03). The results are consistent with the ATPases in which both mutants partially relieve the inhibition by troponin IT in the absence of Ca^{2+} (Dobrowolski et al., 1991) and allow greater relief than wild type of troponin I inhibition in the absence of Ca^{2+} (Figure 7b). dSEEE–pyrene–troponin I interaction was similar to wild type in the absence of Ca^{2+} but in its presence resulted in a much higher monomer/excimer ratio than that of wild type (0.69 ± 0.07; Figure 8b). Thus, the structure of troponin I in the troponin I–C complex with this mutant is altered even though it functions normally. The two remaining mutants, dEDA and dKG, were indistinguishable from wild type in their interaction with pyrene–troponin I.

We also measured the affinity of the troponin Cs for pyrene–troponin I in the presence of saturating Ca^{2+}. The association constants of mutant and wild-type troponin Cs were similar, about (2–3) × 10^7 M^{-1}, with the exception of dKG, which may be slightly lower (results not shown).

Together, analysis of the mutant troponin Cs using these two assays for troponin I–C interaction shows that the functional defects of two of the mutants, dKGK and dKEDAKGK, can be explained by altered binding to troponin I in conditions in which the high-affinity sites are unoccupied or occupied by Ca^{2+} or Mg^{2+}. The functional differences are not due to altered affinity for Ca^{2+} or troponin I. The inability of dEDA and dKG to fully activate in the presence of Ca^{2+} cannot be fully explained by altered troponin I interaction. However, the greater inhibition by dEDA of a regulated system in the

absence of Ca^{2+} may be attributable to altered binding to troponin I.

Discussion

We have carried out more extensive analysis of five troponin C central helix deletion mutants in order to understand the molecular basis for the functional phenotypes observed in actomyosin and actomyosin S1 ATPases (Dobrowolski et al., 1991) and troponin C depleted skinned muscle fibers (Sheng et al., 1991).

All mutants bind Ca^{2+} to both low- and high-affinity sites and undergo Ca^{2+}-dependent conformational changes as evidenced by changes in electrophoretic mobility, α-helix content, hydrophobic exposure, and fluorescence [this paper and Dobrowolski et al. (1991)]. However, the conformations of the mutants detected using this methods differ from each other and from wild type in ways that cannot obviously be related to the "designed" changes for the mutants or to the observed functional defects. For example, dSEEE, which differs from wild type in α-helix content, cation-dependent increase in α-helix, hydrophobic exposure, and the magnitude of the change in the pyrene–troponin I monomer/excimer ratio, is indistinguishable from wild type in all functional assays. The structural differences observed in free troponin C must not be important for the functions we have assayed. Troponin C may be able to compensate for the differences upon binding to other thin filament proteins, consistent with the idea that it is a highly adaptable protein and that the central helix is a flexible structure (Perschini & Kretsinger, 1988). [For a discussion of the relationship between these troponin C central helix mutants and other central helix mutants in calmodulin and troponin C, see Dobrowolski et al. (1991).]

We have made progress in understanding the functional defects of the central helix mutants in terms of their associations with the other troponin subunits. Of the four mutants that have altered regulatory function, three differ from wild type in troponin I interaction only when the high-affinity sites are occupied by Ca^{2+} or Mg^{2+}, the "relaxed" state. In troponin I–C complexes, as well as in a reconstituted thin filament, dKGK and dKEDAKGK partially convert troponin I to the "active" state as evidenced by greater relief of troponin I inhibition of the actomyosin S1 ATPase and the increase in the monomer/excimer ratio of pyrene–troponin I relative to wild type. In contrast, dEDA inhibits the ATPase in the absence of Ca^{2+} to a greater extent than wild type does in a fully reconstituted regulatory system (Dobrowolski et al., 1991). It may be expected that altered regulatory function in the absence of Ca^{2+} relates to interaction with troponin I since troponin C binds strongly to troponin T only in the presence of Ca^{2+}.

The two mutants altered in regulatory function in the presence of Ca^{2+}, dEDA and dKG, function normally with troponin I with Ca^{2+}. The defects of these mutants must be in the ternary troponin complex (or on the regulated thin filament) in interaction with troponin T, directly or via troponin I.

Interestingly, the Ca^{2+} dependence of the fluorescence change in pyrene–troponin I–troponin C complexes is the same for all mutants. Therefore, the different troponin Cs in the complexes all have the same Ca^{2+} affinity for the low-affinity sites. The apparent Ca^{2+} affinity for the low-affinity sites of troponin C alone is also similar for all troponin Cs (Dobrowolski et al., 1991). Although binding of troponin C to troponin I increases the Ca^{2+} affinity to the low-affinity sites (Potter & Gergely, 1975), the effect is the same for all mutants.

On the other hand, the Ca^{2+} dependence of the actomyosin S1 ATPase or the force development in reconstituted skinned fibers of dEDA, dKG, and dKGK differs from wild type, depending on the assay (Dobrowolski et al., 1991; Sheng et al., 1991). Other thin filament proteins, as well as myosin heads, can change the Ca^{2+}-binding properties of troponin C (Bremel et al., 1972; Potter & Gergely, 1975). The present results show that alterations in the central helix are sensitive to these interactions at a higher order of assembly than that of the troponin I–C complex.

Acknowledgments

We thank Dr. D. Winkelmann for the gift of myosin S1, Dr. A. Shatkin for the use of his HPLC, and Mrs. J. Caponigro for assistance in manuscript preparation.

Registry No. Ca^{2+}, 7440-70-2; Mg^{2+}, 7439-95-4.

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