Cloning and Expression of Salmon Cardiac Troponin C: Titration of the Low-Affinity Ca^{2+}-Binding Site Using a Tryptophan Mutant†

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Received March 22, 1996; Revised Manuscript Received June 6, 1996⊗

ABSTRACT: Activation of cardiac actomyosin ATPase requires the occupation of the single low-affinity Ca^{2+}-binding site of troponin C (cTnC). Previously, we demonstrated pronounced differences between mammals and cold-water salmonid fish in the Ca^{2+} sensitivity of cardiac preparations, particularly in relation to temperature [Churcotte, C., Moyes, C. D., Baldwin, K., Bressler, B., & Tibbits, G. F. (1994) *Am. J. Physiol. 267*, R62−R70]. In this study, we examine the extent to which cTnC structure could account for the observed differences in myofibrillar Ca^{2+} sensitivity. Salmonid (*Oncorhynchus mykiss*) cTnC was cloned, sequenced, and expressed in *Escherichia coli* as a maltose-binding protein fusion. The coding region has 87% homology with human cTnC cDNA and differs in 13 of 161 amino acid residues from the human/bovine/porcine isoform. The sequence corresponding to the single regulatory Ca^{2+}-binding site II is completely homologous to that of mammals. The protein expressed exhibits optical properties similar (circular dichroism, intrinsic fluorescence) to those of cTnC purified from salmonid (*Salmo salar*) and bovine ventricle. A single tryptophan residue was introduced into the inactive Ca^{2+}-binding site I (ScTnC-FW27) to facilitate Ca^{2+} titration. The Ca^{2+}-binding constant ($K_{1/2}$ = 5.33 pCa units) was within the range reported for the low-affinity sites of mammalian cTnC. Although differences in TnC primary structure are striking, Ca^{2+} affinity of intact cardiac myofibrils is likely influenced by interactions with other troponin proteins.

Troponin C (TnC)[1] is the Ca^{2+}-binding protein of vertebrate striated muscle myofibrils that is responsible for the regulation of contraction. It has a helix−loop−helix structural motif typical of many Ca^{2+}-binding proteins, including calmodulin, parvalbumin, and myosin light chain (Marsden *et al*., 1990; Collins, 1991). The skeletal muscle isoform (sTnC) possesses two Ca^{2+}-binding sites of relatively low affinity (sites I and II, $K_d \sim 10^{-5}$ M) and two sites of high affinity (sites III and IV, $K_d \sim 10^{-7}$ M). In contrast, cardiac isoforms of TnC have a single functional low-affinity Ca^{2+}-binding site and two sites of high affinity [reviewed by Grabarek *et al*. (1992); Farah & Reinach, 1995]. James and co-workers (Herzberg *et al*., 1986) proposed a model for the changes in the conformation of sTnC that accompany Ca^{2+} binding, which has been extended to cTnC (Putkey *et al*., 1989; Brito *et al*., 1991; Krudy *et al*., 1992). At resting intracellular Ca^{2+} concentrations (∼100 nM; Bers, 1991), the high-affinity sites are expected to be occupied. The structure that is induced in the high-affinity sites by steady state occupancy with either Ca^{2+} or Mg^{2+} is thought to promote anchoring of TnC into the troponin complex (Potter & Gergely, 1975; Zot & Potter, 1982). At resting intracellular Ca^{2+} concentrations, the low-affinity site(s) is unoccupied. The increase in cytosolic free Ca^{2+} concentration accompanying each contraction causes the low-affinity site(s) of TnC to become occupied, with a concomitant reorganization of helices in the protein: helices B and C move, as a unit, away from helices A and D. A hydrophobic patch is exposed and new interactions between TnC and TnI are made possible. In turn, these events are associated with the reorientation of the troponin complex in the thin filament and the release of inhibition of myofibrillar ATPase. Actomyosin ATPase activity persists until Ca^{2+} is resequestered from the cytosol, the low-affinity site(s) is vacated, and tonic inhibition by troponin is reestablished. The physiological relevance of the HMJ model has been well supported by recent studies (e.g., Fujmori *et al*., 1990; Grabarek *et al*., 1990; Gagne *et al*., 1994).

The primary structure of TnC is highly homologous among vertebrates (Collins *et al.*, 1991). However, physiological studies reveal pronounced interspecies differences in the Ca^{2+} sensitivity of myofibrils in both skeletal (e.g., Stephenson & Williams, 1981, 1985; Godt & Lindley, 1982) and cardiac muscles (e.g., Harrison & Bers, 1990a; Churcotte *et al*., 1994). The influence of factors such as temperature (Stephenson & Williams, 1981, 1985; Harrsion & Bers, 1990a; Churcotte *et al*., 1994), pH (Blanchard *et al*., 1984; Solaro *et al*., 1989), and sarcomere length (Babu *et al*., 1988; Moss *et al*., 1988) are highly dependent upon species and developmental stage. As the temperature decreases, cardiac myofibrils become less sensitive to Ca^{2+} [reviewed by Tibbits *et al*. (1993)]. The temperature dependence of myofibrillar Ca^{2+} sensitivity resides, at least in part, with the TnC isoform (Harrison & Bers, 1990b). Animals with low body temper-

† Funded through operating grants from NSERC-Canada (T.J.B., G.F.T.), Heart and Stroke Foundation of BC and Yukon (G.F.T.), British Columbia Health Research Fund (T.J.B.), and fellowships from MRC-Canada (C.D.M.).

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⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1996.

[1] Abbreviations: A_{600}, absorbance at 600 nm; CD, circular dichroism; MBP, maltose-binding protein; PCR, polymerase chain reaction; PFU, plaque-forming units; Tn, troponin.

S0006-2960(96)00705-2 CCC: $12.00 © 1996 American Chemical Society

atures (e.g., cold-water fish) appear to compensate for the desensitizing effects of low temperature with a myofibrillar apparatus inherently more sensitive to Ca^{2+} than that of mammals (see Churcotte *et al*., 1994). Cardiac myofibrils from cold-water fish also show greater temperature-dependent changes in Ca^{2+} sensitivity.

In this study, we examined the contribution of cTnC structure to the interspecies differences in Ca^{2+} sensitivity of the myofibril. We cloned and sequenced cTnC from a cold-water salmonid[2] (*Oncorhynchus mykiss*). A mutant was constructed with a single tryptophan residue introduced into the inactive Ca^{2+}-binding site. The mutant protein facilitates fluorescence studies of Ca^{2+} binding. Similar mutants have been used to titrate TnC Ca^{2+}-binding sites (Trigo-Gonzales *et al*., 1992; Pearlstone *et al*., 1992; Chandra *et al*., 1994).

EXPERIMENTAL PROCEDURES

Restriction endonucleases and DNA-modifying enzymes were purchased from Bethesda Research Laboratories and New England Biolabs. Oligonucleotides were synthesized on an Applied Biosystems Model 392 DNA/RNA synthesizer. Sequencing was performed by using the chain termination method, with T7 DNA polymerase (Sequenase) obtained from U.S. Biochemicals.

Construction of the cDNA Library. Rainbow trout (*Oncorhynchus mykiss*, 400−600 g) were held in outdoor freshwater ponds (∼10 °C) under natural photoperiod. Ventricular tissue was collected, and poly(A)+ RNA was purified as previously described (Tibbits *et al*., 1992). The cDNA library was constructed with the Pharmacia "Time Saver" cDNA synthesis kit using 3.3 mg of poly(A)+ RNA. cDNAs were fitted with *Eco*RI adapters, ligated into Lambda Zap II, and then grown in *Escherichia coli* strain XL1-Blue. Titer before amplification was approximately 10^5 plaque-forming units (PFU)/mL and was amplified to 3×10^9 PFU/mL. Nine of ten randomly selected clones possessed *Eco*RI inserts.

Screening of the cDNA Library. The library was screened by using as probe an *Eco*NI fragment (468 bp) from a mutant chicken sTnC [FW29 from Trigo-Gonzales *et al*. (1992)]. This probe codes for a region of the protein spanning helices B−G. The agarose gel-purified *Eco*NI fragment was radiolabeled by using a random priming method (Feinberg, 1991). Screening of the cDNA library was done on 90 mm NZCYM plates at a density of approximately 3000 PFU/plate. Phage DNA was fixed (Stratagene UV Stratalinker) onto Zeta-Probe nylon membranes and hybridized according to Ausubel *et al*. (1992) at 42 °C. Phages that proved to be positive upon a second screening were converted to single-stranded phagemids by in vivo excision with R408 helper phage. Infection of fresh XL1-Blue with phagemids provided double-stranded plasmids (pBluescript). Inserts were subcloned and sequenced in pUC18 (New England Biolabs). Both sense and antisense strands of the gene were sequenced in their entirety.

Expression Vector. Forward and reverse PCR primers were constructed for the TnC coding region in such a way as to allow the introduction of a unique restriction site. The forward primer (P1) contained a single-nucleotide mismatch (silent) at nucleotide 21 (A to C) to create a unique *Sac*II site: (P1) 5′-ATG AAC GAC ATC TAC AAA GCC GCG G-3′. The reverse antisense primer (P2) included a 5′-tail with a *Xba*I, *Sal*I site: (P2) 5′-CCC GTC GAC TCT AGA TTA TTC TAC TCT TTT CAT GAA CTC-3′.

The coding region of TnC was amplified by 30 PCR cycles using vent polymerase, 0.2 mM dNTPs, 20 ng/mL pBluescript-ScTnC, and 12 μg/mL of each primer at an annealing temperature of 51 °C. The amplified region was digested with *Xba*I and directionally ligated into the vector pMALc (New England Biolabs) which was previously cut with *Stu*I and *Xba*I. The resulting plasmid (pMALc-ScTnC) was used to transform *E. coli* strain TB1, and the plasmid was resequenced by using two overlapping internal primers.

Site-Directed Mutagenesis. Mutagenesis of ScTnC was carried out to replace the phenylalanine in position 27 with a tryptophan residue. An oligonucleotide (P3) was constructed in which the phenylalanine codon 27 (TTT) was replaced with TGG: (P3) 5′-GCC TTT GAC ATC TGGATC CAG GAT GCG-3′. PCR (30 cycles, 54 °C annealing temperature) was conducted by using vent polymerase with 20 ng/mL template (pMALc-ScTnC), 12 μg/mL reverse primer (P2), and 400 ng/ml mutant primer (P3). Because there is excess reverse primer relative to the mutant primer, both single-stranded and double-stranded products were made. A range of P3 concentrations (20-400 ng/mL) was tested to determine the lowest amount of primer that yielded a maximum amount of single-stranded product. After PCR, the sample was electrophoresed on a 2% agarose gel. The single-stranded product was excised from the gel and purified. The single-stranded DNA was used as a reverse primer (P4) in a second PCR reaction with P1 (12 μg/mL each primer). The double-stranded DNA product from this second reaction was then used as a template for the PCR reaction involving the original primers (P1, P2), as described for wild-type TnC. The product was digested with *Sac*II and *Xba*I, gel-purified, and ligated into the wild-type plasmid (pMALc-ScTnC), which had been similarly digested and treated with calf intestinal phosphatase. The resultant plasmid (pMALc-ScTnC-FW27) was used to transform *E. coli* strain TB1 and to express a maltose-binding protein (MBP)-ScTnC-FW27 fusion (as described for the wild-type ScTnC). The mutation was confirmed by sequencing. Unless stated otherwise, transformants used in the expression of wild-type and mutant TnC were grown in 2× TY broth or 0.7% agar plates that contained 200 μg of ampicillin/mL.

Expression of Recombinant Salmonid cTnC. MBP-ScTnC was purified by ion exchange and hydrophobic chromatography because the fusion protein did not bind to the affinity resin provided by the manufacturer. No effort was made to alter the conditions to allow the use of the affinity column, as conventional methods of chromatography proved satisfactory. Frozen glycerol stocks of TB1 cells containing the pMAL-ScTnC fusion plasmid were plated and grown overnight. A single colony was picked and grown in 4 mL of 2× TY broth plus ampicillin to a cell density giving an absorbance at 600 nm (A_{600}) of 0.6. This culture served as an inoculum for a 200 mL broth culture which was then used as an inoculum (at A_{600}=0.6) for a 16 l broth culture grown

[2] The terms "salmon" and "salmonid" derive from the family Salmonidae. This family includes both Atlantic and Pacific salmon (*Salmo* sp. and *Oncorhyncus* sp., respectively). More detail on the choice of species is available in Experimental Procedures. Rainbow trout was previously considered an Atlantic salmon (*Salmo gairdneri*) and was only recently renamed as a Pacific salmon (*Oncorhyncus mykiss*).

in a 20L fermentor. Cells were grown to $A_{600} = 0.6$, and IPTG (isopropyl 1-thio-β-D-galactoside) was added to a final concentration of 0.3 mM to induce expression of the fusion protein MBP-ScTnC. After 2 h, the cells were harvested, lysed, and frozen (−20 °C) as previously described (Trigo-Gonzales *et al*., 1992).

Purification of Fusion Proteins and Recombinant ScTnC. Lysates were thawed by the addition of 1 vol of low-salt column buffer A [8 M urea, 50 mM Tris (pH 8.0), 2 mM $MgCl_2$, 10 mM β-mercaptoethanol, 85 mM KCl]. Urea (solid) was added to give a final concentration of 8 M. The solution was loaded onto a 2.5 × 40 cm Q-Sepharose column (Pharmacia), which had been preequilibrated with low-salt column buffer A. The column was washed with 500 mL of buffer A and then eluted with a linear KCl gradient from 85 to 500 mM (for MBP-ScTnC) or 1 M (for cII-ScTnC). Peak fractions were pooled and desalted on a G-25 Sephadex column (Pharmacia) (2.5 × 100 cm) equilibrated with 0.2% trifluoroacetic acid. Protein fractions were pooled, lyophilized, and stored at −20 °C until processed. Fusion proteins were digested as follows. Approximately 200 mg of lyophilized protein was dissolved in factor Xa buffer (50 mM Tris, 2 mM $CaCl_2$, pH 8.0) and digested at 37 °C with 800 ng of Factor Xa [purified according to Trigo-Gonzales *et al.*, (1992)] added in four aliquots, one every 2 h, with the digest left overnight at room temperature following the final addition. After digestion, solid NaCl was added to 200 mM, and the samples were centrifuged (10 min at 10000*g*), degassed, and applied directly to a Pharmacia Mono-Q (HR 10/10) column equilibrated in column buffer B (50 mM Tris, 2 mM EDTA, 2 mM dithiothreitol, 0.1 mM PMSF, pH 8.0) with 200 mM NaCl. The MBP did not bind to the column, whereas the ScTnC was eluted in a linear salt gradient (0.5%/min) at 300 mM salt. The ScTnC fractions were applied directly to a phenyl Sepharose column (1 × 15 cm) equilibrated in buffer C (50 mM Tris, 15 mM $CaCl_2$, 1 mM DTT, pH 7.5) containing 500 mM NaCl. The column was washed with 100ml buffer C containing 500 mM NaCl and again with 100 mL of buffer C alone. Recombinant ScTnC was eluted with buffer D (50 mM Tris, 15 mM EDTA, 1 mM DTT at pH 7.5), dialyzed twice against 100 vol water, lyophilized and stored at −20 °C.

Purification of Native Salmonid and Bovine cTnC. Rainbow trout cardiac tissue was used to construct the cDNA library because this species is a common model for physiological studies. For economic reasons, native ScTnC was purified from the closely related salmonid species *Salmo salar*, available through local commercial fish-processing facilities. Approximately 1 kg of Atlantic salmon hearts (∼500 fish) was collected, and the ventricles were frozen on dry ice and stored at −80 °C. Bovine heart was collected from a local slaughterhouse and stored at −20 °C. Crude troponin was extracted from tissues essentially as described by Potter (1982) to the stage of a 30−50% ammonium sulfate precipitation. This precipitate was dissolved in low-salt column buffer A and dialyzed overnight in 100 vol of buffer A. The sample was loaded onto a 2.5 × 25 cm Q-Sepharose column and eluted as described for MBP-ScTnC. ScTnC was eluted from the column between 300 and 350 mM NaCl. Fractions were pooled and dialyzed as described earlier for the recombinant proteins. Flocculant material was removed by centrifugation. Subsequently, the proteins were precipitated in 30−50% ammonium sulfate. This precipitate was redissolved in low-salt column buffer A and dialyzed twice against 1000 vol of buffer C containing 500 mM NaCl. cTnC was purified on a 2.5 × 20 cm phenyl Sepharose column as described for the recombinant protein. Fractions of cTnC were pooled and precipitated with 50% ammonium sulfate. The pellet was resuspended in low-salt column buffer A, dialyzed against 5 mM ammonium bicarbonate, and stored frozen at −80 °C prior to purification by FPLC. Final purification was by FPLC on Mono-Q as described for recombinant TnC. Fractions were pooled, dialyzed against water, freeze-dried, and stored at −20 °C.

Spectroscopic Studies. Each protein was dissolved in 6 M urea to a concentration of 20 mg of lyophilized protein/mL. Samples were immediately diluted with an equal volume of Chelex-100-treated ("Ca-free") buffer E [50 mM MOPS, 100 mM NaCl, 1 mM EGTA (added after Chelex-100 treatment), pH 7.00] and dialyzed against 500 vol of the same buffer with three changes. Proteins were removed from dialysis bags, flash-frozen, and stored at −80 °C until required for spectroscopic studies. All plasticware used for the preparation of Ca^{2+}-free proteins was rinsed with 6 N HCl prior to use.

Circular dichroism studies were performed at 21 °C using a Jasco J710 spectropolarimeter. Samples were diluted to 10 μM with buffer E in a cuvette with a 1 mm path length. Spectra were obtained in the absence of Ca^{2+} and after the addition of $CaCl_2$ to 2 mM (pCa = 3.2).

Fluorescent studies were performed on a Photon Technology Inc. LS-100 luminescence spectrofluorometer. Samples were diluted to 5 μM with buffer E in a quartz cuvette (1 cm^2). Spectra were obtained at 21 °C using an excitation wavelength of 276 nm. Titration of the tryptophan mutant was at an emission wavelength of 330 nm and at both 305 and 330 nm for the native salmon protein. Free Ca^{2+} concentrations were determined by using MaxChelator (Bers *et al*., 1993).

RESULTS AND DISCUSSION

Amino Acid and cDNA Sequence Homologies. Comparison of TnC primary structure reveals remarkable conservation across the vertebrate species studied to date (Collins, 1991). Although the ScTnC isoform described in the present study shares features with the other isoforms, it is much less homologous. At the nucleotide level (Figure 1), the 483-bp coding region shows between 80 and 87% sequence identity with mammalian (86.6%, human; 84.3% mouse) and avian (82.9%, chicken) cTnC cDNA sequences. Weaker homologies occur with sTnC isoforms (rabbit, 70.3%; quail, 81.8%). Amino acid sequence of TnC isoforms is highly conserved within birds and mammals, with two or fewer amino acid differences between any species studied to date. ScTnC differs in 13 positions from human/bovine/porcine and 14 positions from chicken cTnC isoforms.

Sequence Differences in Relation to Ca^{2+}-Binding Sites. Despite a high degree of sequence homology in TnC isoforms, myofibrillar Ca^{2+} sensitivity determined in vitro shows considerable phylogenic and ontogenic variability (Churcotte *et al*. 1994; Harrison & Bers, 1990a). In this study, we focused on the structural properties that may account for the interspecies differences in binding properties of cTnC isoforms. A 10^7-fold range of Ca^{2+} affinities is apparent when surveying members of the family of Ca^{2+}-

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ATG AAC GAC ATC TAC AAA GCA GCG GTA GAG CAG CTG ACA GAC GAG CAG AAA AAC GAG TTC 060
... G.T ... ... ..T ..G ..G ... ..T ... ... T.. ... ..A ..A ..A ... ..T ... ..T

AAA GCG GCC TTT GAC ATC TTT ATC CAG GAT GCG GAG GAC GGC TGC ATC AGT ACC AAG GAG 120
..G ..T ... ..C ... ... ..C G.G .T. .GG ..A ... ..T ... ... ... ..C ... ... ...

CTG GGC AAG GTG ATG AGG ATG CTG GGG CAG AAC CCC ACC CCC GAG GAG CTG CAG GAG ATG 180
... ..G ... ... ... ... ... ... ... ... ... ... ... ..T ... ... ... ... ... ...

ATC GAT GAG GTG GAT GAA GAT GGC AGT GGA ACT GTG GAC TTT GAT GAG TTC CTG GTG ATG 240
..T ... ... ... ... ..G ... ... ... ..C ... ... ... ... ... ... ... ... ..T ..T ...

ATG GTG AGG TGC ATG AAG GAC GAC AGC AAA GGG AAA ACA GAG GAG GAA CTG GCA GAT CTC 300
... ..T C.. ..T ... ..A ..T ... ... ... ..A ... ..T ..A ... ..G ..C T.. ... ...

TTC TGC ATG TTT GAC AAG AAT GCG GAC GGC TAT ATA GAC CTG CAG GAG CTG AAG GTA ATG 360
... A.G ... ... ..T ... ... ..T ..T ... ..C ..C ..T ..T G.. ..A ... ... A.C ...

CTG GAG GCC ACA GGG GAG GCC ATC ACT GAG GAC GAC ATT GAG GAG CTC ATG AAG GAC GGA 420
..A C.. ..A ..T ..A ... A.G ... ... ... ..T ... ..A ..A ..A ..G ... ..A ..T ..G

GAC AAG AAC AAC GAT GGG AAA ATC GAC TAC GAC GAG TTT TTA GAG TTC ATG AAA GGA GTA 480
... ..A ... ..T ... ..C .GG ..T ... ..T ... ... ..C C.G ... ... ... ..G ... ..T

GAA TAA      Salmon cardiac troponin C
... ...      Bovine cardiac troponin C
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FIGURE 1: Nucleotide sequence of the coding region of salmonid (*Oncorhynchus mykiss*) cTnC in comparison to bovine cDNA.

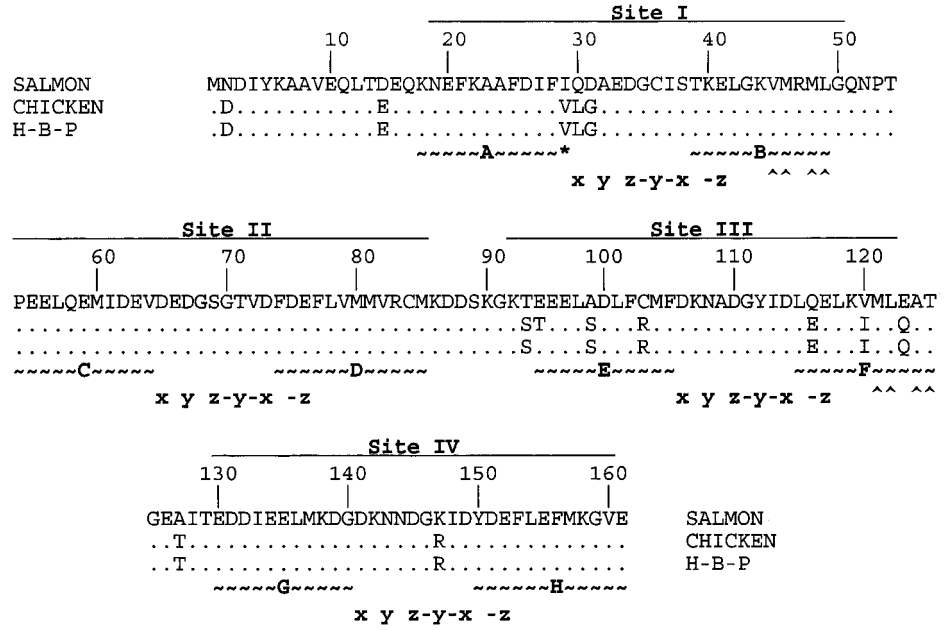

FIGURE 2: Amino acid sequence of salmonid (*Oncorhynchus mykiss*) cTnC (ScTnC) derived from the nucleotide sequence in Figure 1. Regions of identity with sequences determined for chicken cTnC and human/bovine/porcine (HBP) cTnC are denoted by dots. Ca^{2+}-binding sites I−IV are based on analogous regions in the Herzberg, Moult, and James (1986) model for sTnC. Secondary structures are reported for comparison in the context of the HMJ model. Helices A−H are denoted by ∼, and coordinating residues and *x*, *y*, *z*, −*y*, −*x*, −*z*. The asterisk symbol denotes the amino acid insertion that contributes to the inactivation of site I. The carat symbols refer to the location of residues forming the hydrophobic patches in low- and high-affinity domains.

binding proteins with parvalbumin ($K_d = 10^{-9}$ M) and aequorin ($K_d = 10^{-2}$ M) at the extreme ends of the continuum (Marsden *et al*., 1990). Even when comparing the individual ion-binding sites within a single TnC isoform there can be a 10^2-fold range in Ca^{2+} affinities. Differences in the ion-binding affinity of TnC isoforms can result from structural changes both inside and outside the binding loops. For example, Ca^{2+} affinity of the high-affinity sites can be altered as much as 0.5 pCa unit by single amino acid substitutions in the N-cap position of helix G (Trigo-Gonzales *et al*., 1992). The mechanism that underlies differences in Ca^{2+} sensitivities of myofibrils may be related to either TnC affinity per se or how binding-induced structural changes in TnC are interpreted by other elements of the thin filament, notably TnI. Ca^{2+} sensitivity is affected by TnI and TnT isoforms (Wattanapermpool *et al*., 1995; Akella *et al*., 1995), as well as by TnI phosphorylation (Zhang *et al*., 1995). It is important to recognize that the biochemical strategies for altering Ca^{2+} sensitivity over evolutionary time may fundamentally differ from those used to alter Ca^{2+} sensitivity over physiological time.

In the canonical Ca^{2+}-binding loop there are six residues involved in ligation of the ion. The six residues sit at positions around the ion that approximate the axes of a Cartesian coordinate system (i.e., at positions *x*, *y*, *z*, −*y*, −*x*, −*z*). Only one of the sequence differences observed between fish and higher vertebrates (Figure 2) occurs in a Ca^{2+}-coordinating residue (*x*, *y*, *z*, −*y*, −*x*, −*z*). The −*y* position of site IV is occupied by arginine in all sTnC and cTnC isoforms of birds and mammals, but by lysine in ScTnC. A lysine occurs in this position of frog sTnC and parvalbumin Ca^{2+}-binding sites (Marsden *et al*., 1990): it

may represent the "ancestral" Ca^{2+}-binding site (Collins, 1991).

Cardiac isoforms of TnC are unable to coordinate Ca^{2+} at site I because of amino acid substitutions and insertions in the loop relative to the sequence of sTnC. In cTnC isoforms sequenced prior to our study, binding loop I is disrupted in every case by a valine insertion preceding residue *x* and replacement of *x* and *y* with uncharged residues (van Eerd & Takahashi, 1976). In ScTnC, there is an isoleucine insertion prior to *x* as opposed to the valine of higher vertebrates. Although there are three substitutions in the ScTnC loop I that introduce charges into the loop region, none occur at coordinating positions and it is unlikely that loop I is a functional Ca^{2+}-binding site. This does strictly not preclude an influence of site I differences on Ca^{2+} binding by site II. Gulati and Rao (1994) found that the basis of tissue-specific differences in myofibrillar activation by Sr^{2+} resides in the inactive Ca^{2+}-binding site of cardiac isoform. When the cardiac nonfunctional Ca^{2+}-binding site was converted to the functional skeletal analogue, the protein took on the Sr^{2+} activation properties of the skeletal isoform. It is not known whether the modest site I differences appearing between cardiac isoforms could similarly influence Ca^{2+} binding by site II. The sequence identity over the remainder of the regulatory domain (residues 31−92) suggests that interspecies variation in the functional Ca^{2+} affinity of the myofibrils is dependent upon differences elsewhere, either the nonregulatory regions of the TnC sequence or the target regions of TnI.

Sites of Interaction between TnC and TnI. The cation sensitivity of intact myofibrils does not depend solely upon differences in the ion affinity of TnC. Although a study by Gulati and Rao (1994) attributes the Sr^{2+} activation phenotypes of skeletal and cardiac myofibrils entirely to differences in TnC site I sequence, interactions between TnC and the other components of the troponin complex clearly influence Ca^{2+} sensitivity of the myofibril. The $K_{1/2}$ for Ca^{2+} changes profoundly when TnC is complexed with intact TnI (e.g., Solaro *et al*., 1989). TnI inhibitory peptides similarly influence the binding properties of the two structural binding sites (Van Eyk *et al*., 1991; Chandra *et al*., 1994), although the effects on the regulatory sites are ambiguous [Van Eyk *et al.* (1991) vs Chandra *et al*. (1994)]. Developmental TnI isoforms alter the pH sensitivity of Ca^{2+}-binding properties of mammalian cTnC (Solaro *et al*., 1988). The single amino acid difference between the inhibitory regions of s- and cTnI appears to have a marked effect on the Ca^{2+} affinity of s- and cTnC (Van Eyk *et al*., 1991). Each of these studies illustrates the ability of TnC−TnI interactions to influence either TnC Ca^{2+} binding per se or functional Ca^{2+} sensitivity of the myofibrils.

Several regions of the low-affinity domain have been implicated in interactions with TnI. Farah *et al.* (1994) suggest that TnC and TnI are arranged antiparallel, with structural domains of TnC (C-terminal) and TnI (N-terminal) associated when sites III and IV are occupied at rest. When Ca^{2+} is bound by the low-affinity site(s) of TnC, the regulatory regions (N-terminal TnC, C-terminal TnI) interact more strongly. Several sites of interaction between TnC and TnI, both Ca-dependent and Ca-independent, have been identified (see Farah & Reinach, 1995). Although potential differences in TnI target regions cannot be ignored, cTnC sequence comparisons (Figure 2) suggest that interactions

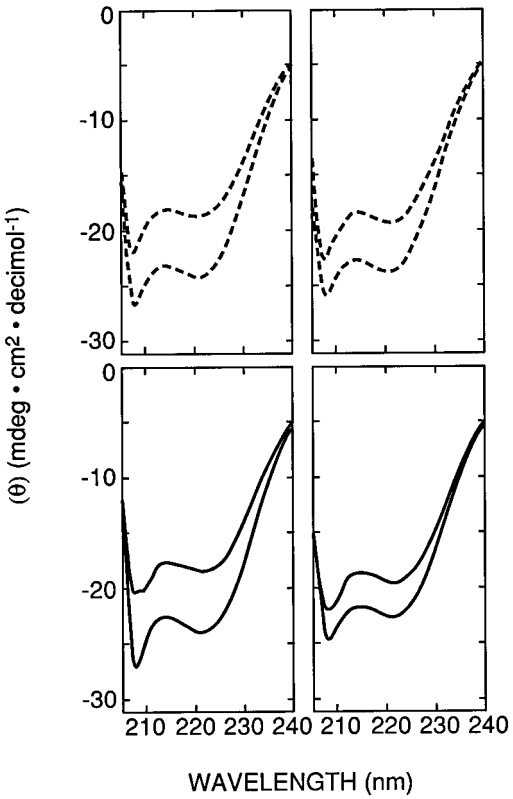

FIGURE 3: Far-UV circular dichroism spectra for cTnC from recombinant rainbow trout (*Oncorhynchus mykiss*) wild-type ScTnC (upper left), Atlantic salmon (*Salmo salar*) heart (upper right), tryptophan mutant ScTnC-FW27 (lower left), and bovine heart (lower right). Lower curves in each panel represent Ca^{2+}-free conditions. Upper curves are the spectra in the presence of saturating Ca^{2+} (pCa = 3).

with the N-terminal region of TnC do not account for interspecies differences in Ca^{2+}sensitivity. The hydrophobic patch (Herzberg *et al*., 1986) and other N-terminal regions of TnC implicated in interactions with TnI (Leszyk *et al*., 1990; Wang *et al*., 1990; Kobayashi *et al*., 1991) are highly conserved across species. Perhaps the most striking differences between salmon and higher vertebrates are the series of substitutions in the high-affinity domain, particularly in the D/E linker and helix E. Early studies by Grabarek *et al*. (1981) and Dalgarno *et al*. (1982), and more recently Leszyk *et al*. (1987, 1988), show interactions with TnI and this region of TnC.

Spectroscopic Studies. Circular dichroism and intrinsic fluorescence were used to compare the native and recombinant ScTnC's to the well-characterized human/bovine/porcine isoform (BcTnC). When assayed under identical Ca^{2+}-free conditions, all four proteins exhibited similar far-UV CD spectra (Figure 3). The change observed with Ca^{2+} binding was similar in each salmon protein (Table 1), although BcTnC demonstrated less of a decrease in ellipticity. Tyrosine fluorescence spectra for native bovine and ScTnC virtually overlap, whereas that of recombinant ScTnC exhibited a slight shoulder at higher wavelengths (Figure 4). The reason for this difference in tyrosine fluorescence spectra is not known.

Titrations were performed on recombinant ScTnC for both wild type and FW27. Fluorescence spectra of the proteins exhibited expected peaks at 305 nm for the wild-type (tyrosine) and 330 nm for the tryptophan mutant (ScTnC-FW27). When spectra were obtained at equal protein

Table 1: Molar Ellipticity Changes (222 nm) of Cardiac Troponin C Isoforms in Response to Ca^{2+}

species	source	Θ (deg cm^2/dmol^{-1})[a]
O. mykiss (ScTnC, wild type)	recombinant	5.5
O. mykiss (ScTnC-FW27)	recombinant	5.5
S. salar	hearts	4.4
bovine	heart	3.1

[a] Molar (residue) ellipticity is based on a 0.18 mg/mL solution, calculated molecular weight of 18 000, 161 residues, and path length of 0.1 cm.

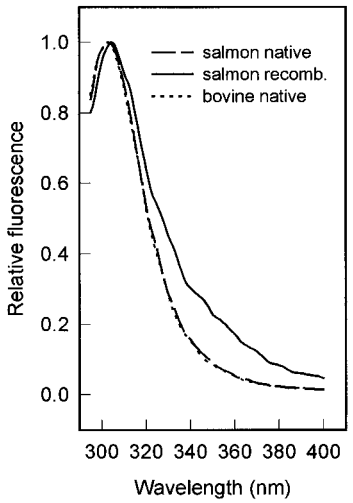

FIGURE 4: Fluorescence spectra for Ca^{2+}-loaded (pCa = 3) cTnC from bovine heart (B), native salmon (*Salmo salar*) heart (SN), and recombinant rainbow trout (*Oncorhynchus mykiss*) wild-type ScTnC (SR). Excitation was at 276 nm. More details are available in Experimental Procedures.

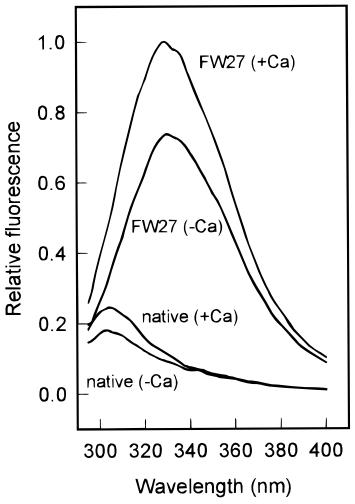

FIGURE 5: Titration of fluorescence of salmonid recombinant ScTnC and ScTnC-FW27. Representative spectra were determined with equimolar concentrations of each protein (5 μM). For each protein, the upper curve is Ca^{2+}-saturated (pCa = 3.2) TnC.

concentrations, it was apparent that the contribution of tyrosine residues to the overall fluorescence of the spectral mutant was not significant (Figure 5). Furthermore, titration of the wild type (305 nm) showed complex kinetics dominated by changes in the high-affinity domain of the protein where two of the three tyrosines reside. At 330 nm, the wavelength chosen for titration of ScTnC-FW27, the fluorescence of the wild type was half that at 305 nm. These observations support the premise that the fluorescence changes in the wild type at 330 nm do not substantially affect

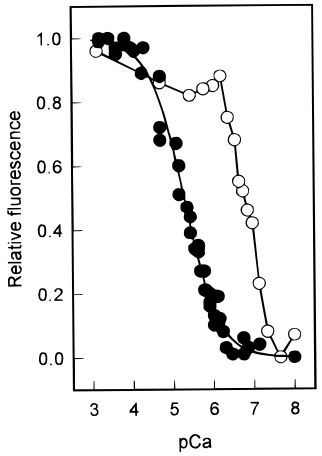

FIGURE 6: Titration of fluorescence of salmonid recombinant ScTnC and ScTnC-FW27. Each protein was titrated with Ca^{2+} at 21 °C, pH 7.00. For wild-type recombinant ScTnC (10 μM) the emission wavelength was 305 nm. For tryptophan mutant ScTnC-FW27 (5 μM), no correction was made for the minor contribution of intrinsic tyrosine fluorescence expected based on wild-type protein titrations. Emission wavelength was 335 nm for ScTnC-FW27.

the changes observed upon titration of ScTnC-FW27. Ca^{2+} was found to bind to the regulatory Ca^{2+}-binding site with a $K_{1/2}$ of pCa = 5.33 (Figure 6). Ca^{2+} affinity of the regulatory site of mammalian cTnC has been determined by a number of methods. Attempts to titrate this site in BcTnC by intrinsic tyrosine fluorescence and circular dichroism are complicated by the fact that most of the effect of Ca^{2+} binding occurs in the high-affinity domain with both techniques (Hincke *et al*., 1978; Van Eyk *et al*., 1991). A value of $K_{1/2}$ = 5.7 pCa was reported for the low-affinity sites of BcTnC, based upon a combination of CD and fluorescence (Van Eyk *et al.*, 1991). This is within the same range as that reported in this study for ScTnC, given the differences in assay conditions and relative sensitivities of spectral parameters. When cysteine residues are in convenient locations fluorescent probes can be attached covalently, although the steric effects of introducing a bulky probe into TnC structure are not clear. As cardiac isoforms have multiple cysteine residues (two in human/bovine/porcine, three in salmon), systematic protection of particular residues would be necessary and likely result in heterogeneous labeling. Tryptophan mutants have been used to advantage with Ca^{2+}-binding proteins such as sTnC (Pearlstone *et al*., 1992; Trigo-Gonzales *et al*., 1992) and calmodulin (Chabbert *et al*., 1992). Ca^{2+} affinities of a series of tryptophan mutants determined by fluorescence (Pearlstone *et al*., 1992) are similar to those determined directly by Ca^{2+} binding (da Silva *et al*., 1993). Although tryptophan mutants have been used previously in proteins lacking tyrosine residues, allowing unambiguous assignment of fluorescence, the presence of tyrosine residues in ScTnC does not appear to complicate the titration. In this particular case, tryptophan fluorescence is suitable for the same reason tyrosine fluorescence is less than optimal for titration of the wild-type protein: low quantum yield from tyrosine residues that are responsive to a different range of Ca^{2+} concentrations (Figure 4).

It is noteworthy that the tryptophan substitution occurs in the inactive site I, yet it detects changes occurring in site II upon Ca^{2+} binding. A similar relationship has been shown with a cardiac−skeletal chimera tryptophan mutant (Gulati

& Rao, 1994). The assumption that inactivation of site I does not fundamentally alter the Ca^{2+}-dependent changes in tertiary structure predicted by the HMJ model is supported by studies examining Ca^{2+}-induced changes in the environments of phenylalanine residues of helices in the N-terminal domain of cTnC (Brito *et al*., 1992). Differences between s- and cTnC in the stability of the β-sheet region connecting low-affinity sites may account for the cTnC-specific relationship between sites I and II in response to Ca^{2+} binding (Krudy *et al*., 1992).

In summary, ScTnC exhibits pronounced differences in primary structure relative to the mammalian and avian species. However, the amino acid sequences surrounding the single functional low-affinity Ca^{2+}-binding site of human/bovine/porcine and salmon isoforms are fundamentally similar, predicting comparable apparent Ca^{2+} affinities when isolated TnC is used. The interspecific differences in functional Ca^{2+} sensitivity apparent with myofibrillar studies may manifest only upon interaction with TnI. Such interactions may be influenced by the identified differences in TnC high-affinity domains (D/E linker, helix E) or by as yet unidentified interspecific differences in TnI.

ACKNOWLEDGMENT

The authors gratefully acknowledge the important contributions of Dr. G. Kalmar, S. Sidhu, and K. Racher and the technical assistance of H. Kashihara.

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BI9607057