Amino Acid Sequences and Ca^{2+}-Binding Properties of Two Isoforms of Barnacle Troponin C†

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ABSTRACT: Much of our knowledge of the relationship between elevation of free sarcoplasmic [Ca^{2+}] and skeletal muscle contraction has come from physiological studies on barnacle muscle fibers. Little is known, however, about the biochemical properties of the barnacle proteins responsible for Ca^{2+} regulation. In order to help rectify this unfortunate situation, we purified the two major isoforms ($BTnC_1$ and $BTnC_2$) of troponin C (TnC) from the giant barnacle, *Balanus nubilis*, and determined their amino acid sequences. $BTnC_2$, the more abundant isoform, contains 151 amino acid residues and has a calculated molecular weight of 16838. Due to an elongated N-terminus, $BTnC_1$ contains 158 amino acid residues and has a calculated molecular weight of 17984. The two sequences can be aligned from their C-termini, with no insertions or deletions, but they are only 61% identical. Sequence differences are twice as frequent in the N-terminal halves as in the C-terminal halves but occur in all regions of the polypeptides. This indicates that $BTnC_1$ and $BTnC_2$ are products of different genes, rather than alternative transcripts of a single gene. Both isoforms contain the usual four ancestral Ca^{2+}-binding regions, numbered I–IV from the N-terminus. Analysis of the sequences predicts that functional Ca^{2+}-binding sites are present only in regions II and IV and that these sites are low-affinity Ca^{2+}-specific type sites. Direct Ca^{2+}-binding measurements using fluorescent Ca^{2+} indicators show that both isoforms bind 2 Ca^{2+}/mol with equal affinity ($K_{Ca} = 1.3 \times 10^5$ M^{-1}) and the sites appear to be Ca^{2+}-specific.

It has been known for many years that elevation of free sarcoplasmic [Ca^{2+}] is required for skeletal muscle activation and that there are receptors for Ca^{2+} on the muscle thin filaments, which are responsible for initiating the events that lead to contraction. Much of our knowledge of the first event has come from physiological studies that related free [Ca^{2+}] changes in barnacle muscle fibers to the time course of force development [e.g., Ashley and Ridgway (1970), Ashley (1978), Ashley et al. (1976), Ashley and Moisescu (1977), Griffiths et al. (1984), Jackson et al. (1987), and Timmerman and Ashley (1986)]. The barnacle is ideally suited for this type of study since it has large single striated muscle fibers that can be injected with Ca^{2+}-sensitive indicators and readily voltage clamped. Unfortunately, however, little is known about the biochemical properties of the barnacle proteins responsible for Ca^{2+} regulation. Previously, Potter et al. (1986) were the first to report the isolation and characterization of barnacle troponin and tropomyosin, neither of which regulated actomyosin ATPase activity by itself but which did so when combined. Later, Ashley et al. (1987) briefly reported the purification and properties of major ($BTnC_2$)[1] and minor ($BTnC_1$) isoforms of barnacle troponin C (TnC), the Ca^{2+}-binding component of the muscle thin-filament regulatory complex which also includes troponins I and T (TnI and TnT). TnC is the key regulatory protein that acts as a molecular switch in response to the efflux of Ca^{2+} from the sarcoplasmic reticulum that follows nervous stimulation. In this report we describe a method for purification of barnacle troponin subunits, present the results of sequence analysis of $BTnC_1$ and $BTnC_2$, and describe the Ca^{2+}-binding properties of these two proteins.

TnC belongs to a large family of homologous proteins that includes calmodulin, myosin light chains, and parvalbumins. [For a recent review, see Strydnaka and James (1989).] Early comparative sequence studies (Collins, 1974, 1976a, 1976b; Collins et al., 1973) on these proteins suggested that they evolved from a common ancestor and contained four similar Ca^{2+}-binding regions which arose by gene duplication and reduplication. Each of the regions, designated I–IV from the N-terminus (Collins et al., 1973), consisted of a pair of helices and a central, 12-residue Ca^{2+}-binding site. Evolutionary changes in the amino acid sequences of many present-day proteins have resulted in loss of Ca^{2+}-binding ability while maintaining many features of their three-dimensional structures (Collins, 1974, 1976a,b). As more protein sequences have become available [e.g., see Baba et al. (1984) and Boguta et al. (1988a)], these proposals have been repeatedly confirmed and extended to include an ever-growing list of additional Ca^{2+}-binding proteins with diverse functions. The predicted structural features of TnC turned out to be remarkably accurate, as shown by the crystal structures of chicken (Sundaralingam et. al., 1985; Satyshur et al., 1988) and turkey (Herzberg & James, 1985, 1988) skeletal muscle TnCs. This was fortunate, since a large number of structure-function studies, carried out before the crystal structures became available, were based on the predicted structure (Leavis and Gergely, 1984).

The crystallographic studies show that the TnC molecule

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[1] Abbreviations: TnC, troponin C; $BTnC_1$, barnacle TnC_1; $BTnC_2$, barnacle TnC_2; CNBr, cyanogen bromide; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; PTC, phenylthiocarbamyl; HPLC, high-performance liquid chromatography; TPCK, *N*-tosylphenylalanyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

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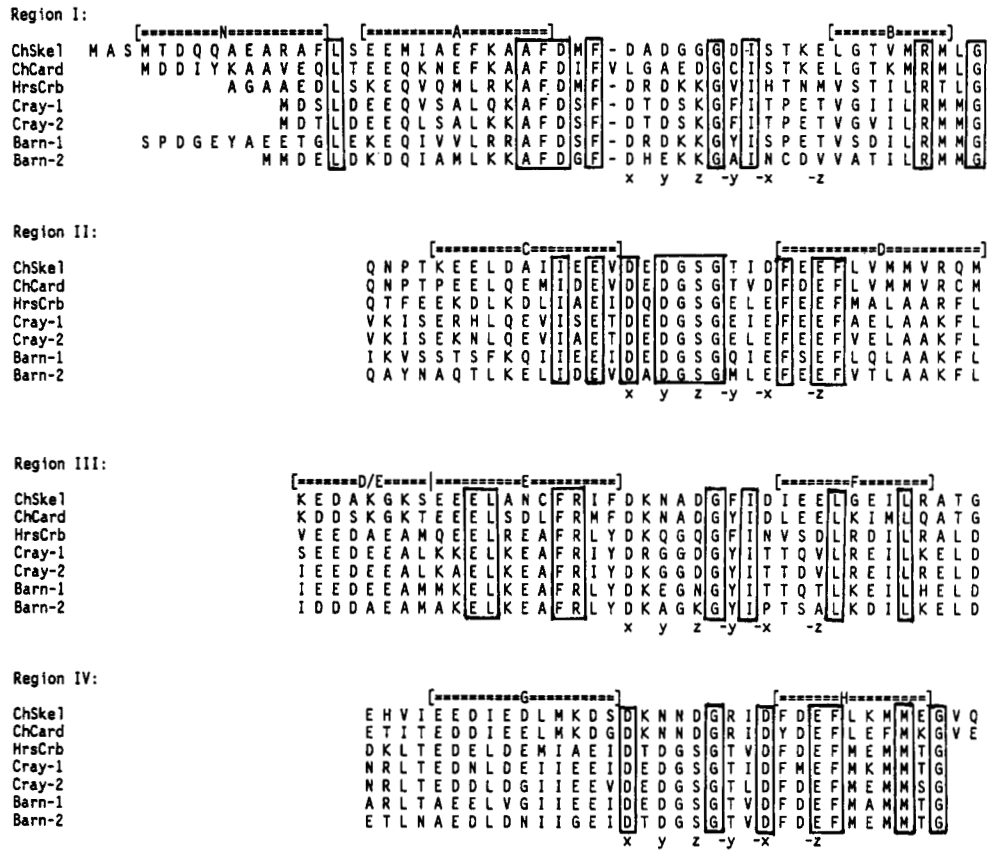

FIGURE 1: Alignment of the amino acid sequences of TnC from several sources. ChSkel, chicken fast skeletal muscle (Wilkinson, 1976); ChCard, chicken cardiac/slow skeletal muscle (Putkey et al., 1987); HrsCrb, horseshoe crab (Kobayashi et al., 1989b); Cray-1 and Cray-2, the α and γ isoforms, respectively, of crayfish (Kobayashi et al., 1989a); Barn-1 and Barn-2, barnacle TnC_1 and TnC_2 (this report). Amino acid residues that are identical in all seven TnC sequences are boxed. The four homologous regions I–IV (Collins et al., 1973) are aligned with each other. Within each region, x, y, z, −y, −x, and −z represent potential Ca^{2+}-coordinating oxygen ligands arranged octahedrally in a 12-residue binding site. The locations of helices, designated N, A, B, C, D–D/E–E, F, G, and H from the N-terminus, observed in the crystal structures of chicken (Satyshur et al., 1988) and turkey (Herzberg & James, 1988) TnCs are shown.

has the general shape of a dumbbell, with two similar, independent domains in the N-terminal and C-terminal halves. As predicted, each domain contains a pair of Ca^{2+}-binding sites, and each site contains a similar helix–loop–helix motif. Residues at positions 1, 3, 5, 7, 9, and 12, numbering from the beginning of each loop, are designated X, Y, Z, −Y, −X, and −Z, as they are capable of binding a Ca^{2+} ion with an approximately octahedral arrangement of oxygen ligands. Consistent with early suggestions, the four Ca^{2+}-binding sites are commonly designated I–IV (Collins et al., 1973) and the expected helices are designated A–H (Collins, 1976b). In addition to the predicted helices, the crystal structure of TnC reveals an N-terminal helix, and also a helical segment, the D/E linker, which is contiguous with helices D and E and connects the two halves of the molecule. These structural features are summarized in Figure 1. The locations of helices A–H are very close to those predicted on the basis of homology. The predicted locations of the Ca^{2+}-binding sites (Collins et al., 1973) have proven to be absolutely correct, although only sites III and IV, the high-affinity Ca^{2+}–Mg^{2+} sites, are occupied in the crystal structure. The unoccupied sites I and II are the low-affinity, Ca^{2+}-specific sites that are responsible for regulation of muscle contraction (Potter & Gergely, 1975). In the crystal structure of calmodulin (Babu et al., 1985), which is very similar to that of TnC, all four expected Ca^{2+}-binding sites are occupied.

TnCs from vertebrate skeletal and cardiac muscles have been thoroughly characterized. Vertebrate fast skeletal muscle TnCs bind four Ca^{2+}, at two low-affinity ($K_{Ca} \sim 10^5\ M^{-1}$) Ca^{2+}-specific sites and two high-affinity ($K_{Ca} \sim 10^7\ M^{-1}$) sites that also bind Mg^{2+} (Potter & Gergely, 1975). Because of mutations that led to critical sequence changes, site I of vertebrate cardiac and slow skeletal muscle TnCs no longer binds Ca^{2+}, and one of the low-affinity sites is lost (van Eerd & Takahashi, 1976). The few invertebrate TnCs that have been sequenced to date all seem to have retained functional Ca^{2+}-binding sites only in regions II and IV (Kobayashi et al., 1989a,b). Although reports of invertebrate TnCs that bind only a single Ca^{2+} have appeared from time to time [e.g., Wnuk et al. (1984)], all TnCs sequenced to date appear to have retained at least two functional sites, located in regions II and IV.

Experimental Procedures

Ca^{2+} Binding Measurements. $BTnC_1$ and $BTnC_2$ were desalted by dissolving the lyophilized proteins in 0.2 M EDTA (pH 7.0, adjusted with KOH), followed by chromatography on a 88 × 1.2 cm Sephadex G-25 column equilibrated with 50 mM NH_4HCO_3. Protein-containing fractions were lyophilized and redissolved in the buffer used for Ca^{2+} binding measurements: 100 mM MOPS and 150 mM KCl, pH 7.0. Ca^{2+} contamination was determined by atomic absorption spectroscopy using a Perkin-Elmer 3030 atomic absorption spectrophotometer. The usual Ca^{2+} contamination of our protein samples varied between 1 and 10 μM. Protein concentration was determined by the Lowry method (Lowry et al., 1951) and adjusted to be between 40 and 80 μM.

The fluorescent Mg^{2+} indicator Mag-Indo-1 (Tsien, 1980) has proven to be a very good Ca^{2+} indicator as well; it is especially suitable for determining the Ca^{2+}-binding properties

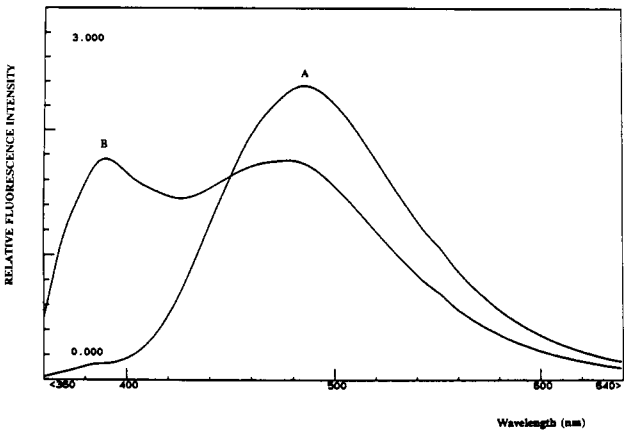

FIGURE 2: Mag-Indo-1 fluorescence emission spectra. Mag-Indo-1 (0.84 μM) was dissolved in a buffer of 150 mM KCl, 100 mM MOPS, and 2 mM EGTA (pH 7.0). Curve A: pCa > 10.0. Curve B: pCa = 3.0. Excitation wavelength: 340 nm.

of low-affinity Ca^{2+}-binding sites, since its dissociation constant for Ca^{2+} is in the micromolar range (K_D = 5 μM, when measured in a Ca^{2+}–EGTA buffer at pH 7.0). Upon binding of Ca^{2+}, the fluorescence emission spectrum of Mag-Indo-1 undergoes spectral modifications (Figure 2). In the metal-free form, Mag-Indo-1 has one major emission peak at 490 nm (Figure 2, curve A). Upon binding of Ca^{2+}, the fluorescence intensity of Mag-Indo-1 at 390 nm increases about 15 times and the peak at 490 nm decreases. The increase in fluorescence intensity at 390 nm was used to calibrate the dye as well as to determine the free Ca^{2+} concentration in solutions of $BTnC_2$.

Fluorescence titrations were performed either on a Perkin-Elmer 650-10S fluorescence spectrophotometer and recorded on a Perkin-Elmer R 100A recorder on a SLM 8000 fluorometer. The concentration of Mag-Indo-1 and Fluo-3 in the sample cuvette was set to be lower than 0.5 μM in order to avoid interference with the Ca^{2+}–protein equilibrium.

Mag-Indo-1 and Fluo-3 (Minta et al., 1989) were purchased from Molecular Probes. Mag-Indo-1 was excited at 340 nm, and fluorescence emission was recorded at 390 nm. Fluo-3 was excited at 505 nm, and emission was recored at 595 nm. Free Ca^{2+} was calculated according to

$$[Ca^{2+}] = K_d(F - F_{min})/(F_{max} - F) \quad (1)$$

where F is the fluorescence emission intensity and F_{max} and F_{min} are, respectively, the values obtained for the Ca^{2+}-saturated and Ca^{2+}-free forms of the dye [from Grynkiewicz et al. (1985)]. In practice, since the protein solution is not completely free of Ca^{2+}, F_{min} is not directly measured but rather is calculated as a function of F_{max}. According to our calibration curves, $F_{min} = 0.066F_{max}$ for Mag-Indo-1 and $F_{min} = 0.046F_{max}$ for Fluo-3.

Calibration of the Ca^{2+} indicators was achieved by setting the free $[Ca^{2+}]$ by using either EGTA or EDTA (2 mM) in a buffer of 100 mM MOPS and 150 mM KCl, pH 7.0, and measuring fluorescence intensity. The stability constants needed to calculate the free $[Ca^{2+}]$ were taken from Martell and Smith (1974). The results were fitted to eq 1 by using the computer program Asystant. K_ds of ~5 μM and 463 nM were found for Mag-Indo-1 and Fluo-3, respectively. Titrations were performed in a 1-cm-square cell in 1–2 mL of $BTnC_1$ or $BTnC_2$ solution maintained at 25 °C. Ca^{2+} (5 mM) was added in aliquots of 2 μL. The amount of Ca^{2+} bound was calculated from the equation

$$[Ca^{2+}]_{bound} = [Ca^{2+}]_{total} - [Ca^{2+}]_{free(equilibrium)}$$

where $[Ca^{2+}]_{total}$ is $[Ca^{2+}]_{added} + [Ca^{2+}]_{contamination}$ and $[Ca^{2+}]_{free}$ is calculated according to eq 1. The amount of Ca^{2+} bound was calculated from data points where the free Ca^{2+} values were within one log unit of the K_d of the indicator used. The results were fitted with the computer program Asystant according to the equation

$$y = (n_iK_i[Ca^{2+}])/(1 + K_i[Ca^{2+}])$$

where y is the number of moles of Ca^{2+} bound per mole of Ca^{2+}-binding protein, n is the number of sites, and K is the Ca^{2+} affinity constant for these sites. Both n and K were allowed to float.

Sequence Strategies. The amino acid sequences of $BTnC_1$ and $BTnC_2$ were determined by protein chemistry methods, well-established procedures described in previous publications from our laboratory (Collins et al., 1986, 1988; Leszyk et al., 1987, 1988). As with most other muscle proteins, the N-termini of $BTnC_1$ and $BTnC_2$ were blocked, and no information could be obtained from direct sequence analyses of the intact proteins. The N-terminal blocking group of $BTnC_1$ was shown to be acetyl by ^1H NMR analysis. The N-terminal blocking group of $BTnC_2$ was not identified but was presumed to be acetyl by analogy with $BTnC_1$ and with TnC from other species. The amino acid sequences of $BTnC_1$ and $BTnC_2$ were reconstructed from the sequences of overlapping peptides produced by cleavage with cyanogen bromide, trypsin, chymotrypsin, *Staphylococcus aureus* V8 protease, and pepsin and by dilute acid hydrolysis. Details can be found in the supplementary material, which is included with the microfilm edition of this paper (see paragraph regarding supplementary material at end of paper).

Results and Discussion

The amino acid sequence of $BTnC_1$ (158 amino acid residues) and $BTnC_2$ (151 residues), aligned with several other representative TnC sequences, are shown in Figure 1. Both $BTnC_1$ and $BTnC_2$ have blocked N-termini, and if they are presumed to be acetylated, their calculated molecular weights at neutral pH are 17984 and 16838, respectively. The $BTnC_1$ and $BTnC_2$ sequences can be aligned from their C-termini without any insertions and deletions but are only 61% identical. The differences occur in all parts of the polypeptides, indicating that $BTnC_1$ and $BTnC_2$ are products of different genes rather than alternative transcripts of a single gene. The differences are not uniformly distributed, however: 39 occur in the N-terminal halves and 20 occur in the C-terminal halves.

As noted previously (Kobayashi et al., 1989a), sequence comparisons have revealed three main areas of variability in TnC. The first is the N-terminal helix, which is shorter or absent in invertebrate TnCs and also in calmodulin. The second area of variability is the D/E linker, which joins the two halves of TnC as the central part of the long helix D–D/E–E. Site-directed mutagenesis of the long helix (Reinach & Karlsson, 1988; Xu & Hitchcock-DeGregori, 1988; Dobrowolski et al., 1990; Sheng et al., 1990) has shown that some major changes can be tolerated without significant functional effect, although others can influence the transmission of structural changes in TnC to other thin-filament proteins. The third area of sequence variability is helix F, in which there are significant differences between vertebrate and invertebrate TnCs. Of the four homologous regions of TnC, region IV is the least conserved, suggesting that evolutionary constraints are less important in the C-terminal half of TnC. It is noteworthy, however, that, in contrast to the overall picture among all TnCs, the two isoforms of barnacle TnC are much more similar to each other in region IV than in region II.

The $BTnC_1$ and $BTnC_2$ sequences are only about 35% identical with those of vertebrate and protochordate TnCs, about 40% identical with calmodulin sequences, and 50-60% identical with other available arthropod TnC sequences from horseshoe crab and crayfish. Judging from the degrees of sequence difference among the arthropod TnC sequences, the divergence of chelicerates (horseshoe crab) from crustaceans (crayfish, barnacle) preceded the divergence of crayfish from barnacle by only a relatively short period, and this was rather quickly (on an evolutionary time scale) followed by the divergence of $BTnC_1$ and $BTnC_2$. It is particularly interesting to compare $BTnC_1$ and $BTnC_2$ with the only other available crustacean TnC sequences, those of two crayfish isoforms, which are 86% identical with each other (Kobayashi et al., 1989a). In contrast to the asymmetric distribution of differences in barnacle TnC, the 23 variable residues in crayfish TnC are evenly distributed throughout their sequences.

Wnuk (1984) originally reported the existence of a single isoform of crayfish TnC, which had only one Ca^{2+}-binding site of physiologically relevant affinity ($K_{Ca} \sim 2 \times 10^5$ M^{-1}). However, more recently (Wnuk, 1989), crayfish TnC has been resolved into at least five isoforms. The two major isoforms (α and γ) of crayfish TnC were purified, and their Ca^{2+}-binding properties were investigated more thoroughly. Isolated α-TnC contains one site with $K_{Ca} \sim 1 \times 10^6$ M^{-1} and one with $K_{Ca} \sim 1 \times 10^4$ M^{-1}; when α-TnC is complexed with TnI or with TnI plus TnT, the K_{Ca} of both sites increases to (2–4) $\times 10^6$ M^{-1}. Isolated γ-TnC contains 2 Ca^{2+}-binding sites of $K_{Ca} \sim 2 \times 10^4$ M^{-1}; when γ-TnC is complexed with TnI or with TnI plus TnT, the K_{Ca} of one site increases to (4–5) $\times$ 10^6 M^{-1}, while that of the other site increases only to $\sim 5 \times$ 10^4 M^{-1}. Mg^{2+} does not appear to bind directly to any of these sites. Wnuk (1989) proposed that each isoform contains only a single regulatory Ca^{2+}-specific site whose affinity is increased by forming a complex with TnI. Kobayashi et al. (1989a) sequenced the α and γ isoforms of crayfish TnC and suggested that the higher-affinity Ca^{2+}-binding site of the α isoform, which is not present in the γ isoform, is located in region IV. They then concluded that region II in both isoforms must contain the site whose affinity is increased by binding to TnI.

In relaxed muscle, sarcoplasmic [Ca^{2+}] is 100 nM or less and TnI binds to actin, causing inhibition of actomyosin ATPase activity. At high [Ca^{2+}], Ca^{2+} binds to the low-affinity site(s) of TnC, causing large conformational changes that are transmitted to TnI, resulting in the removal of inhibition and leading to muscle contraction. There have been several attempts over the past 17 years to predict the Ca^{2+}-binding properties of individual Ca^{2+}-binding sites in TnC from empirical comparisons of available sequences and experimental binding studies. In one of the earliest studies, Collins (1976b) proposed the following test: liganding position Y and the following residue must be Asp-Gly for a Ca^{2+}-specific site and Asn-X (where X is not Gly) for a Ca^{2+}-Mg^{2+} site. Later, when sequence information on calmodulin became available, Potter et al. (1977) also suggested that the presence of a Gly following the Y position was critical for the formation of a Ca^{2+}-specific site. Sites II and IV of both barnacle isoforms have the noted Asp-Gly sequence, suggesting that they are both Ca^{2+}-specific sites. This agrees with recent binding studies (Francois et al., 1990), which show that $BTnC_2$ contains two Ca^{2+}-specific sites and no Ca^{2+}–Mg^{2+} sites. Sites I and III of $BTnC_1$ and $BTnC_2$ are expected to bind Ca^{2+} weakly, or not at all, due to insufficient numbers of Asp, Glu, and other potential liganding residues with oxygen-containing side chains.

A recent comparison of functional binding loops of the vertebrate TnCs led Kobayashi et al. (1989a) to propose a more stringent test of sequence differences between Ca^{2+}-specific and Ca^{2+}–Mg^{2+} sites: first, basic residues occur between the X and Y positions in Ca^{2+}–Mg^{2+} sites; second, Ca^{2+}-specific sites have Asp at the Y position, while Ca^{2+}–Mg^{2+} sites have Asn; third, Ca^{2+}-specific sites have the sequence Gly-(Gly/Ser)-Gly following the Y position but Ca^{2+}–Mg^{2+} sites have the sequence X-Asp-Gly, where X is not Gly. Applying these criteria, Kobayashi et al. (1989a) concluded that sites II and IV of both crayfish isoforms are Ca^{2+}-specific sites, and the same conclusion can be reached for sites II and IV of horseshoe crab TnC (Kobayashi et al., 1989b) and also $BTnC_1$ and $BTnC_2$. The sequence test of Kobayashi et al. (1989a) would predict that calmodulin contains four Ca^{2+}–Mg^{2+} sites, but experimental studies (Potter et al., 1977) show that calmodulin contains four Ca^{2+}-specific sites. On the other hand, the simpler test proposed by Collins (1976b) yields identical results for TnC sites and is also valid for calmodulin (Potter et al., 1977).

In addition to proposing that sites I and II are the Ca^{2+}-specific sites in vertebrate TnCs, Collins (1976b) was also the first to suggest that site II is a functionally essential, regulatory Ca^{2+}-specific site that has been conserved in all TnCs throughout evolution. A large number of studies over the years [see Leavis and Gergely (1984), Zot and Potter (1987), Wnuk (1988), Putkey et al. (1989), and Gulati et al. (1989)] have provided strong support for these ideas.

Boguta et al. (1988a) have published a convenient empirical method for estimating the affinities of Ca^{2+}-binding sites in TnC and related proteins. This method is based on the secondary structure prediction method of Garnier et al. (1978) and takes into account the flanking helices as well as the loops that make up each site. Boguta et al. (1988a) recognized that cooperative effects between the two sites in a domain are critically important in determining binding strength. Both Ca^{2+} in a two-site domain are expected to have the same binding constant. Even if one site is unable to bind Ca^{2+}, it still interacts with the other and moderates its affinity for Ca^{2+}. Application of this method to $BTnC_1$ and $BTnC_2$ predicts that the Ca^{2+} affinity of site IV in both $BTnC_1$ and $BTnC_2$ is quite strong ($K_{Ca} \sim 5 \times 10^6$ M^{-1}). The predicted K_{Ca} of site II is $\sim 5 \times 10^4$ M^{-1} in $BTnC_1$ and $\sim 5 \times 10^5$ M^{-1} in $BTnC_2$. This difference may not be significant, since the estimation method is accurate only to within an order of magnitude (Boguta et al., 1988b). On the other hand, if the difference is real, it could be physiologically relevant, since there is a variable distribution of $BTnC_1$ and $BTnC_2$ in different barnacle muscles (Potter et al., 1986).

Direct Ca^{2+}-binding measurements were performed on $BTnC_2$ by using the fluorescent indicator Mag-Indo-1 (Tsien, 1980). A typical example of Ca^{2+} binding to $BTnC_2$ measured by the Mag-Indo-1 method is shown in Figures 3 and 4. Analysis of the data shows that $BTnC_2$ contains 2.1 ± 0.3 sites with $K_{Ca} = 1.3 \times 10^5$ M^{-1}. No difference in affinity between the two sites was observed. Ca^{2+} binding to $BTnC_1$ was investigated by using another Ca^{2+} indicator, Fluo-3 (Minta et al., 1987), and the results showed that $BTnC_1$ also binds two Ca^{2+}, with affinities similar to those of $BTnC_2$. These results were also confirmed by equilibrium dialysis (data not shown).

The selectivities of the $BTnC_1$ and $BTnC_2$ Ca^{2+}-binding sites were not measured directly, but several arguments favor the hypothesis that both sites are Ca^{2+}-specific. As discussed earlier, functional loops II and IV of both $BTnC_1$ and $BTnC_2$ meet the sequence test (Collins, 1976b) for Ca^{2+}-specific sites. A second argument is that to date only two types of helix–loop–helix Ca^{2+}-binding sites are known: a high-affinity ($K_{Ca} \sim 10^7$ M^{-1}) Ca^{2+}–Mg^{2+} type, which also binds Mg^{2+} com-

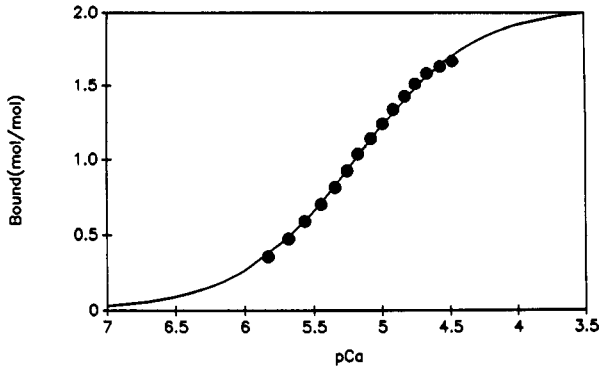

FIGURE 3: Ca^{2+}-binding properties of $BTnC_2$ (80 μM, determined with Mag-Indo-1 (in 150 mM KCl and 100 mM MOPS buffer, pH 7.0). The data points were fitted according to $Y = (nK[Ca^{2+}])/(1 + K[Ca^{2+}])$. The best fit for this experiment was obtained with $n = 2.1 \pm 0.02$ and $K_{Ca} = (1.5 \pm 0.04) \times 10^5$ M^{-1}.

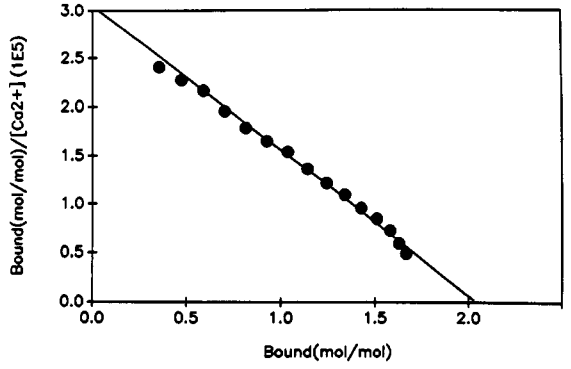

FIGURE 4: Scatchard plot of Ca^{2+} binding to $BTnC_2$. Data are from Figure 3.

petitively ($K_{Mg} \sim 4 \times 10^3$ M^{-1}), and low-affinity ($K_{Ca} \sim 10^5$ M^{-1}) type, which does not bind Mg^{2+} with significant affinity. Since the binding constants of the two sites in $BTnC_1$ and $BTnC_2$ are $\sim 10^5$ M^{-1}, they would seem to belong to the latter, Ca^{2+}-specific type. Finally, a more direct approach has been used to investigate the Ca^{2+} specificity of the $BTnC_2$ sites. $BTnC_2$ was labeled, presumably on Cys-30, with the sulfhydryl-specific probe Acrylodan (Prendergast et al., 1983). Upon binding of Ca^{2+}, the emission spectrum of the labeled protein was shifted to shorter wavelengths. The Ca^{2+} dependence of these changes was not significantly affected by 5 mM Mg^{2+}, showing that at least one, and probably both, of the Ca^{2+}-binding sites do not bind Mg^{2+}.

It is also interesting to note that both $BTnC_1$ and $BTnC_2$ can bind to TnC-depleted skinned barnacle muscle fibers in the presence of Mg^{2+}. Once bound, both isoforms equally activate these fibers in the presence of Ca^{2+} (Ashley et al., 1987). Thus, even though $BTnC_1$ and $BTnC_2$ probably do not contain Ca^{2+}–Mg^{2+} sites, they appear to undergo a Mg^{2+}-dependent association with the fibers. In contrast, $BTnC_1$ and $BTnC_2$ will not activate TnC-depleted rabbit psoas muscle skinned fibers in the presence of Mg^{2+} but will bind and activate them in the presence of Ca^{2+}.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL AVAILABLE

Description of the materials and methods used for protein and peptide purification and of sequencing strategies, and documentation of results in Tables I–IV and Figures 5–27 (43 pages). Ordering information is given on any current masthead page.

Registry No. $BTnC_1$, 130726-95-3; $BTnC_2$, 130726-96-4; Ca, 7440-70-2.

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Molecular Cloning of Rat Cardiac Troponin I and Analysis of Troponin I Isoform Expression in Developing Rat Heart[†,‡]

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ABSTRACT: We have isolated and sequenced a cDNA encoding rat cardiac troponin I. The predicted amino acid sequence was highly identical with previously reported chemically derived amino acid sequences for rabbit and bovine cardiac troponin I. Clones for slow skeletal muscle troponin I were also obtained from neonatal rat cardiac ventricle by the polymerase chain reaction. The nucleotide sequences of these clones were determined to be more than 99% identical with a previously reported rat slow skeletal troponin I cDNA [Koppe et al. (1989) *J. Biol. Chem. 264*, 14327-14333]. The troponin I clones hybridized to RNA from the appropriate muscle from adult animals. However, RNA from fetal and neonatal rat heart also hybridized with the slow skeletal troponin I cDNA, demonstrating its expression in fetal and neonatal rat heart. Slow skeletal troponin I steady-state mRNA levels decreased with increasing age, but cardiac troponin I mRNA levels increased through fetal and early neonatal cardiac development. Thus, during fetal and neonatal development, slow skeletal and cardiac troponin I isoforms are coexpressed in the rat heart and regulated in opposite directions. The degree of primary sequence differences in these isoforms, especially at phosphorylation sites, may result in important functional differences in the neonatal myocardium.

Troponin I (TnI),[1] the inhibitory component of the troponin complex, has an important role in the calcium-dependent regulation of striated muscle contraction [see El-Saleh et al. (1986) and Zot and Potter (1987) for reviews]. As calcium binds to troponin C (TnC), protein-protein interactions occur among TnC, TnT, tropomyosin, and TnI which remove the inhibition of TnI on actin-myosin cross bridging. Chemically determined amino acid sequences for TnI isoforms have been reported from slow skeletal, fast skeletal, and cardiac muscle (Grand et al., 1976; Wilkinson & Grand, 1978; Leszyk et al.,

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[1] Abbreviations: TnI, troponin I; ssTnI, slow skeletal troponin I; cTnI, cardiac troponin I.

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