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Interaction of Troponin C and Troponin C Fragments with Troponin I and the Troponin I Inhibitory Peptide†

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ABSTRACT: We have quantitated the interactions of two rabbit skeletal troponin C fragments with troponin I and the troponin I inhibitory peptide. The calcium binding properties of the fragments and the ability of the fragments to exert control in the regulated actomyosin ATPase assay have also been studied. The N- and C-terminal divalent metal binding domains of rabbit skeletal troponin C, residues 1-97 and residues 98-159, respectively, were prepared by specific cleavage at cysteine-98 and separation by gel exclusion chromatography. Both of the troponin C fragments bind calcium. The calcium affinity of the weak sites within the N-terminal fragment is about an order of magnitude greater than is reported for these sites in troponin C, suggesting interaction between the calcium-saturated strong sites and the weak sites. Stoichiometric binding (1:1) of the troponin I inhibitory peptide to each fragment and to troponin C increased the calcium affinities of the fragments and troponin C. Complex formation was detected by fluorescence quenching or enhancement using dansyl-labeled troponin C (and fragments) or tryptophan-labeled troponin I inhibitory peptide. The troponin C fragments bind to troponin I with 1:1 stoichiometry and approximately equal affinities (1.6×10^6 M^{-1}) which are decreased 4-fold in the presence of magnesium versus calcium. These calcium effects are much smaller than is observed for troponin C. The summed free energies for the binding of the troponin C fragments to troponin I are much larger than the free energy of binding troponin C. This suggests a large positive interaction free energy for troponin C binding to troponin I relative to the fragments. The tryptophan-labeled troponin I inhibitory peptide binds to troponin C and to the N- and C-terminal fragments of troponin C in the presence of calcium with a stoichiometry of 1:1 and association constants of 5.3×10^5, 2.5×10^5, and 6.5×10^4 M^{-1}, respectively. The calcium dependencies of the association constants are largest for troponin C (~10-fold) with smaller values for the N-terminal (~3-fold) and C-terminal (~2-fold) fragments. These data are most easily understood in terms of a solution structure for troponin C which is compact relative to the crystal. The large calcium dependence in the troponin C-troponin I interaction (>50-fold) might arise from the coupling of a conformational change in the N-terminal domain of troponin C with the release of a positive free energy of interaction between the calcium binding domains.

Regulation of the contraction of skeletal muscle is achieved through the dependence of the interactions of the troponin-tropomyosin-actin complex of the thin filament on the presence of calcium ion in the calcium regulatory sites of troponin C (Leavis & Gergely, 1984). An understanding of the molecular basis of this regulation is contingent on a knowledge of the structures and interactions of all the protein components. Structural information is available for troponin C (Herzberg & James, 1985; Sundarlingham et al., 1985), tropomyosin (Caspar et al., 1969; Phillips et al., 1980), and actin (Suck et al., 1981; Engelman & DeRosier, 1983; Kabsch & Holmes, 1990). Although little detailed structural information is known

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for troponin I, troponin T, troponin, or troponin–tropomyosin complexes, general features of their shapes are available [see review by Zot and Potter (1987)]. In troponin, all the subunits are known to interact with each other; tropomyosin interacts with actin, and troponin is bound to tropomyosin via troponin T. Additionally, troponin I interacts with actin directly. Information on the sites of mutual interaction in the primary sequences of these proteins has been summarized (Zot & Potter, 1987). Measurements of the actomyosin ATPase in the presence of various combinations of the troponin subunits and tropomyosin have also revealed many interesting features of the control process (Leavis & Gergely, 1984; Van Eyk et al., 1986).

Experiments to the present clearly support the notion that the troponin–tropomyosin complex is a component of an allosteric switch. Initiation of contraction occurs when calcium ions bind to the regulatory sites of troponin C, the calcium binding subunit of troponin, affecting the interactions between all of the subunits. These changes in interactions are how the signal is transmitted, i.e., how the switch turns on and off. The interaction between troponin C and troponin I shows the largest strength dependence on Ca^{2+} of all the subunit interactions (Ingraham & Swenson, 1984; Wang & Cheung, 1985). Binding of calcium to the regulatory sites of troponin C increases the strength of this interaction and weakens the interaction between troponin I and actin. A primary part of the system's response to the binding of Ca^{2+} to the regulatory sites of troponin C is to change the interaction of troponin C and of actin with a critical site, residues 104–115, on the troponin I inhibitory subunit of troponin (Talbot & Hodges, 1981; Dalgarno et al., 1982; Cachia et al., 1983; Grabarek et al., 1986; Van Eyk & Hodges, 1988). The dumbbell-shaped troponin C molecule is thought to interact with troponin I at three sites (Grabarek et al., 1981); two of the interactions, located near the II_N (51–62) and III_N (89–102) helices, are calcium-dependent, and the third near the IV_N (126–140) helix is independent of calcium ion. The proposed site of interaction for the troponin I inhibitory peptide is residues 89–102 of troponin C (Dalgarno et al., 1982; Cachia et al., 1983; Leszyk et al., 1987, 1988). Some recent experiments suggest that the troponin I inhibitory peptide also interacts with the N-terminal domain near calcium binding site II (Leszyk et al., 1990). The current hypothesis (Talbot & Hodges, 1981; Van Eyk & Hodges, 1988; Grabarek et al., 1986) for the molecular control of muscle contraction is then as follows: In the absence of calcium ion bound to the regulatory sites of troponin C, troponin I interacts with actin, inhibiting the ATPase activity; in the presence of calcium ions in the regulatory sites of troponin C, troponin I interacts with troponin C near the III_N helix, relieving the inhibition.

An interesting feature of the proposed structure of this control complex is that the regulatory calcium ion sites are in the N-terminal domain of the troponin C molecule and the residues of troponin C which interact with the inhibitory-peptide region of troponin I are in the III_N helix near the C-terminal domain. The "signal" that calcium ion is bound must travel from the N-terminal domain to the C-terminal domain of troponin C. As the N-terminal domain of troponin C also interacts with troponin I, this can occur in an allosteric manner through the "fabric" of the troponin I structure, through the α helix connecting the domains of troponin C, or through some combination of both. Earlier studies of the binding of calcium ion to troponin C suggest that transmission could be through the D/E helix of troponin C (Wang et al., 1983; Grabarek et al., 1986). General ATPase studies suggest that the regulation is allosteric; thus, the signal could also be transmitted through troponin I (Green & Eisenberg, 1980).

Recent studies have investigated the importance of interactions of the connecting helix of troponin C to signal transmission and function with deletion and insertion mutants (Xu & Hitchcock-DeGregori, 1988; Dobrowolski et al., 1991a,b). All of the mutant troponin C's were able to confer calcium sensitivity on the ATPase but were defective in some way due likely to altered interaction with troponin I and troponin T. Force generation in skinned fibers was also measured (Sheng et al., 1991). Only one of the mutants, a deletion of two residues, showed significantly altered force development. (Deletion or insertion of a number of residues corresponding to nonintegral numbers of turns causes a rotation of the N- and C-terminal domains with respect to each other.) This two-residue deletion was predicted to cause a 160° rotation in the connecting helix, the largest of any mutant tested. It was suggested that this rotation of domains modulated the function.

In this study, we explore the troponin C–troponin I interaction interface in greater detail with regard to the control mechanism. To this end, we have performed the first direct binding studies of the interaction of the troponin I inhibitory peptide, residues 104–115, with troponin C fragments which are functional domains of troponin C and of the interactions of the fragments with troponin I. We use two fragments of rabbit skeletal troponin C: residues 1–97, the regulatory calcium sites I and II plus the D/E helix, and residues 98–159, the calcium–magnesium sites III and IV. The effects of calcium on these interactions were determined as were the calcium binding properties of each of the troponin C fragments. Measurement of the effects of the troponin I inhibitory peptide on calcium binding to the fragments provided an indirect measurement of the interaction between the troponin C fragments and the troponin I inhibitory peptide. The ability of the fragments individually and combined to support calcium control was assessed in a regulated actomyosin ATPase assay. Our results are interpreted in terms of the crystal structure and other studies which have provided information on the solution structures and interactions in this system.

Materials and Methods

Preparation and Labeling of Troponin C Fragments. Troponin C from rabbit skeletal muscle was cleaved at the single cysteine, residue 98, with 2-nitro-5-thiocyanobenzoic acid (Jacobsen et al., 1973). The protein, at a concentration of 2.5 mg/mL, was dissolved in a solution containing 0.2 M Tris buffer, pH 8, 6 M guanidine hydrochloride, and 0.2 mM dithiothreitol. The reagent (Sigma) was added to 10-fold excess over the sulfhydryl groups in the buffer and troponin C, and the reaction was allowed to proceed for 20 min at 37 °C; at this time, the pH was increased to 9 and the cleavage proceeded for 6 h. The reaction mixture was dialyzed vs water and lyophilized. For separation of the products by gel exclusion chromatography, the reaction mixture was dissolved in a minimal amount of 0.05 M potassium phosphate buffer, pH 7, containing 0.02% sodium azide. Samples (1.5 mL) were loaded onto a 2 × 75 cm G-50 Sephadex column and eluted at 20 mL/h as controlled by gravity fed from a large reservoir. The troponin C fragments were identified by analysis on 15% polyacrylamide in the presence of sodium dodecyl sulfate and reducing agent or by sodium dodecyl sulfate–urea gels following the procedure described by Bio-Rad.

The troponin C fragments were labeled with dansylaziridine following the procedure of Johnson et al. (1978) for troponin C. The N-terminal domain is labeled at methionine-25 as

described (Johnson et al., 1978), and the C-terminal domain is likely labeled at one or more of the methionines.

Synthesis of Troponin I Inhibitory Peptide. The troponin I inhibitory peptides, residues 104–115 with and without tryptophan at position 103, used for the early measurements were synthesized and purified (Talbot & Hodges, 1979) in Robert Hodges' laboratory. We have also synthesized these two peptides using the T-bag method of Houghton (Houghton, 1985; Houghten et al., 1986) following the detailed procedures of Merutka (1990). Both sets of the peptides were acetylated and amidated at the N- and C-termini, respectively. The purity of the peptides was estimated to be >95% from reverse-phase HPLC on C18 columns. Peptide molecular weights were confirmed by plasma desorption mass spectrometry on a Bio-Ion Nordic BIN-10K plasma desorption time of flight mass spectrometer (calculated peptide M_r 1550.95, observed M_r 1551 ± 1; calculated tryptophan peptide M_r 1737.2, observed M_r 1736 ± 1). The composition was confirmed by amino acid analysis. The concentration of solutions of the tryptophan-labeled peptide was determined using an extinction coefficient of 5600 M^{-1} cm^{-1} at 280 nm. The concentration of unlabeled peptide was determined by the Micro BCA* protein assay reagent (Pierce) using the tryptophan-labeled peptide as standard.

Fluorescence Measurements. Fluorescence measurements for determination of the strength of protein–peptide and protein–protein interactions and of calcium binding to troponin C and its fragments were made on an SLM 4800C fluorometer. Macro programs written for specific experiments were used to provide for signal averaging, data storage, and uniformity in the performance of the experiment.

Interactions between Proteins and Peptides. The interaction between the troponin C fragments and troponin C and the troponin I inhibitory peptide were followed by observing the enhancement of the intrinsic fluorescence of tryptophan which was incorporated into the peptide at position 103. The interaction between troponin I and the troponin C fragments was studied using dansylated troponin C fragments. After isolation of the part of the fluorescence signal due to complex formation and correction for dilution, the association constants were obtained from a nonlinear least-squares fit to the equation for binary complex formation:

$$K = \frac{[X]}{([P^*] - [X])([P_i] - [X])} \quad (1)$$

$$F_i = [X]F_{max}/[P^*] \quad (2)$$

where $[P^*]$ and $[P_i]$ are, respectively, the total concentration of the labeled protein and the unlabeled protein after the ith addition. The observed fluorescence, F_i, provides a signal which is proportional to [X], the concentration of the binary complex for the ith addition of unlabeled protein. The parameters of the fit are K, the association constant, and F_{max}, the fluorescence value when all the labeled protein is complexed. The stoichiometry was also used as a parameter to assure that a best fit was for 1:1.

These fluorescence measurements were made at 20 °C in three buffer systems at pH 7.0. The buffers contained 10 mM Pipes, 0.1 or 0.3 M KCl, 3 or 5 mM dithiothreitol, 0.01% NaN_3, and either 1 mM $CaCl_2$ (calcium buffer), 3 mM $MgCl_2$ and 1 mM EGTA (magnesium buffer), or 2 mM EDTA.

Calcium Binding Studies. The binding of calcium to troponin C and its fragments was studied at 24 °C in 20 mM Tris buffer, pH 7.5, which contained 0.1 M KCl and 0.01% sodium azide. The effect of saturating concentrations of the troponin I inhibitory peptide on calcium binding was also measured. Two fluorescence measurements were made for each point in the titration. Fractional saturation was monitored by intrinsic tyrosine fluorescence; there is one tyrosine in each of the fragments. Free calcium ion concentration was monitored by fluorescence of the calcium-sensitive fluors BAPTA or 4,4′-difluoro-BAPTA (Molecular Probes, Inc.). Calcium fluors were chosen to have dissociation constants similar to those being measured. The excitation and emission wavelengths were 255 and 364 nm, 258 and 370 nm, and 282 and 300 nm, respectively, for BAPTA, 4,4′-difluoro-BAPTA, and tyrosine fluorescence. In a typical titration, 1.5 mL of a solution of ~5 μM troponin C or a fragment and 5 μM calcium fluor in buffer which contained sufficient calcium to achieve >99% saturation of all calcium sites was titrated with 1–5-μL aliquots of 2 mM EDTA in the same buffer (pH adjusted). The total volume added was <10% of the sample volume. A macro program was written to run the instrument during the titration. Following each addition from a calibrated syringe microburet, the sample was stirred magnetically for 90 s with the shutters closed. The signal was averaged for 40 s at each set of excitation and emission conditions, and the data were stored. Limiting values for tyrosine fluorescence and BAPTA or difluoro-BAPTA fluorescence were established by initial values in the presence of saturating calcium and when no further change occurred in the presence of excess EDTA (calcium free).

Fractional saturation with calcium ion was calculated following each addition from the tyrosine fluorescence and the limiting values for tyrosine fluorescence. Free calcium concentration after each addition was calculated from the BAPTA or 4,4′-difluoro-BAPTA fluorescence and the corresponding limiting values for their fluorescence in the equation:

$$[Ca^{2+}]_i = K_d(F_f - F_i)/(F_i - F_s) \quad (3)$$

The BAPTA or 4,4′-difluoro-BAPTA dissociation constant is K_d, F_f is the limiting value of the calcium fluor fluorescence in a calcium ion free solution, F_s is the limiting value of fluorescence when the calcium fluor is saturated with calcium ion, and F_i is the measured fluorescence after the ith addition. Corrections for dilution were made to all data prior to analysis.

There are two attractive features of the method we employed to study calcium binding in TnC and its fragments. First, determination of free calcium concentrations and protein fractional saturation depends only on the limiting values of calcium fluor and tyrosine fluorescence, respectively; contaminating calcium is not a problem nor do the concentrations of calcium binding protein and calcium fluor need to be known precisely. Second, the presence of single tyrosine residues within each of the TnC fragments precludes the need for incorporation of extrinsic probes with subsequent probe effects. The choice of calcium fluorophore was dictated by the approximate calcium affinity of the protein under study. Given that the most accurate range of free calcium concentrations corresponds to calcium fluor fractional saturation between 10 and 90%, the protein of interest must titrate in the same range if significant experimental error is to be avoided. Using K_d's measured for our buffer system, these ranges are approximately 5–370 nM for BAPTA and 30–2400 nM for 4,4′-difluoro-BAPTA. On the basis of previously reported calcium affinity constants for the strong (~2.5×10^7 M^{-1}) and weak sites (~2×10^5 M^{-1}) within TnC, BAPTA was chosen for strong site measurements and 4,4′-difluoro-BAPTA for weak site measurements.

Macroscopic calcium dissociation constants for troponin C and the fragments were obtained by fitting these data to the equation assuming two sets of sites for troponin C and a single

set of sites for the troponin C fragments:

$$\theta_i = \theta_a[Ca^{2+}]_i^{N_a}K_a^{N_a}/(1 + [Ca^{2+}]_i^{N_a}K_a^{N_a}) + \theta_b[Ca^{2+}]_i^{N_b}K_b^{N_b}/(1 + [Ca^{2+}]_i^{N_b}K_b^{N_b}) \quad (4)$$

In this equation, θ_i is the fractional saturation after the ith addition and θ_a and θ_b are the fractions of the total fluorescence change associated with each set of sites, with their respective association constants, K_a and K_b, and Hill coefficients, N_a and N_b.

Measurement of BAPTA and 4,4′-Difluoro-BAPTA Dissociation Constants. Dissociation constants for BAPTA and 4,4′-difluoro-BAPTA were measured for our solution conditions with the EGTA/NTA calcium buffering system using the published absolute association constants (Sillen & Martell, 1964). Equations for a given total metal concentration were solved simultaneously for the case of two ligands using the Newton–Raphson procedure (Perrin & Sayce, 1967) to give the free calcium concentration. The fluorescence titrations were performed with 1.5 mL of the following solutions: 1 μM BAPTA or 4,4′-difluoro-BAPTA, and one of the calcium buffer systems, either 1 mM EGTA or 0.5 mM EGTA and 0.5 mM NTA, containing 20 mM Tris buffer, pH 7.5, 0.1 M KCl, and 0.01% sodium azide. This solution was titrated with 10 mM calcium chloride in the same buffer lacking the calcium fluors in which the pH was adjusted to 8.85 to use the buffering capacity of Tris to maintain the pH at 7.5 upon calcium ion binding. The dissociation constants for the calcium fluor's were obtained by fitting the observed calcium fluor fluorescence as a function of the calculated free calcium concentration to a model for binary complex formation:

$$F_i = F_f - (F_f - F_s)[[Ca^{2+}]/(K_d + [Ca^{2+}])] \quad (5)$$

F_i is the fluorescence after the ith addition of calcium ion, F_f and F_s are, respectively, the fluorescence of the calcium fluor in the solution when free and saturated with calcium ion, $[Ca^{2+}]$ is the free calcium ion concentration, and K_d is the dissociation constant.

Regulated Acto–Subfragment 1 ATPase Assays. Actomyosin ATPase assays were performed as previously described (Williams & Swenson, 1982). Fixed-time assays were used wherein the rate of production of phosphate was measured by the method of Fiske and SubbaRow (1925). The solution buffer was adjusted to pH 7 and contained 10 mM imidazole, 30 mM KCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.02% sodium azide, and either 0.1 mM $CaCl_2$ or 1.0 mM EGTA.

Troponin was reconstituted by combining equimolar amounts of troponin I and troponin T with either a 2-fold excess of troponin C or a 3–10-fold excess of fragments in 6 M guanidine hydrochloride. Cycles of buffer exchange, using Amicon Centricon-3 microconcentrators or dialysis, were performed to remove denaturant. Protein concentrations were determined spectrophotometrically at 280 nm using the following extinction coefficients ($E^{1\%,1cm}_{280nm}$): troponin T, 5.04 (Margossian & Cohen, 1973); troponin I, 3.97 (Wilkinson, 1974); troponon C, 1.59 (Leavis et al., 1978); troponin, 4.5 (Hartshonne & Mueller, 1969); troponin C N-terminal fragment, 1.10; troponin C C-terminal fragment, 1.66. The extinction coefficients for troponin C fragments were calculated from the molar extinction coefficient of 1197 M^{-1} cm^{-1} for tyrosine (Fasman, 1976) and the fragment molecular weights with one tyrosine per fragment as is known from the sequence.

The extinction coefficient for the troponin I–troponin T complex was calculated by adding appropriate molecular extinction coefficients for each subunit.

Preparation of Rabbit Skeletal Muscle Proteins. Myosin was prepared by the procedure of Perry and Grey (1956) as modified by Goodno and Swenson (1975). Myosin subfragment 1 was prepared by chymotryptic digestion of insoluble myosin (Weeds & Taylor, 1975). Actin was prepared by the procedure of Spudich and Watt (1971) with added high-salt washes to remove tropomyosin and α-actinin. Troponin and tropomyosin were prepared from the same ether powder used in the preparation of actin following the procedure of Potter (1982) with two changes. The crude troponin was passed over an Affi-gel Blue column (Reisler et al., 1980) before separation into subunits (Ingraham & Swenson, 1984). Pure troponin was obtained by DEAE chromatography of the troponin from the Affi-gel Blue column (Ingraham & Swenson, 1984). Tropomyosin was purified on a hydroxylapatite column (Eisenberg & Kielley, 1974).

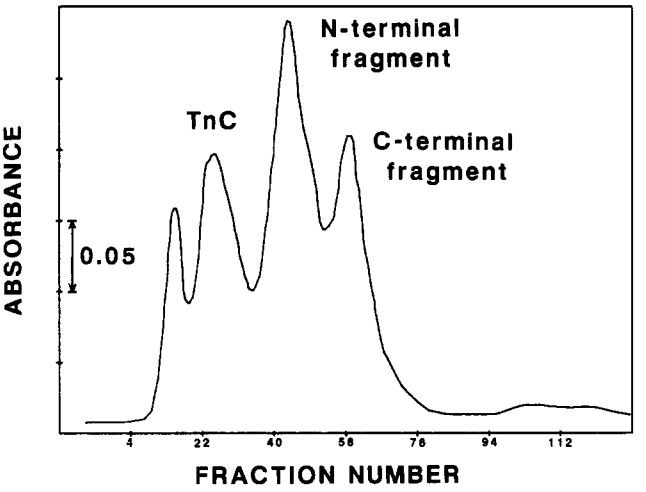

FIGURE 1: Chromatography of cleavage products of troponin C. The digest, in buffer, was concentrated and applied to a G-50 Sephadex column (2 × 75 cm). About 2 column volumes were used to elute the components from the column. The fraction size was 1 mL.

Results

Preparation and Purification of the Troponin C Fragments. Cleavage of troponin C with 2-nitro-5-cyanothiobenzoic acid at cysteine-98 gave the two fragments, 1–97 and 98–159. The former has the usual carboxyl terminus, and the latter has a 2-iminothiazolidine-4-carboxyl N-terminus. The cleavage leaves the N- and C-terminal calcium binding domains intact but is near the C-terminus of the proposed calcium dependent binding site for the troponin I inhibitory peptide, residues 89–100.

The cleavage was specific but not quantitative. In five preparations, the percent conversion ranged from 60 to 80% as estimated from gel electrophoresis; only three components were visible on gels, troponin C and the two fragments. The N-terminal and C-terminal fragments were separated from the parent troponin C by gel exclusion chromatography as is illustrated in Figure 1. In order of appearance, the peaks were troponin C, N-terminal fragment, and C-terminal fragment. The small peak at the void volume of the column contained no protein. In samples where the cleavage reagent was incompletely removed by dialysis, a UV-absorbing peak containing no protein followed the elution of C-terminal fragment but did not overlap it. Appropriate pooling of the fractions yielded relatively pure C-terminal fragment. The pooled fractions containing the N-terminal fragment were contaminated with the C-terminal fragment and troponin C and were rechromatographed to obtain pure N-terminal fragment.

The identity of the N- and C-terminal fragments was consistent with their elution from the size exclusion column and mobility on 15% SDS–PAGE gels and SDS–PAGE–urea gels.

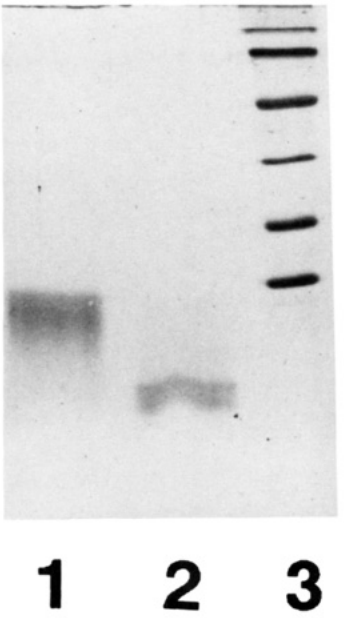

FIGURE 2: 15% SDS–PAGE of purified N- and C-terminal fragments of troponin C. Lane 1, troponin C N-terminal fragment; lane 2, troponin C C-terminal fragment; lane 3, low molecular weight standards (Bio-Rad) containing proteins with molecular masses of 14 400, 21 500, 31 000, 45 000, 66 200, and 92 500 kDa. Samples represent protein pooled after two gel filtration runs over G-50 Sephadex.

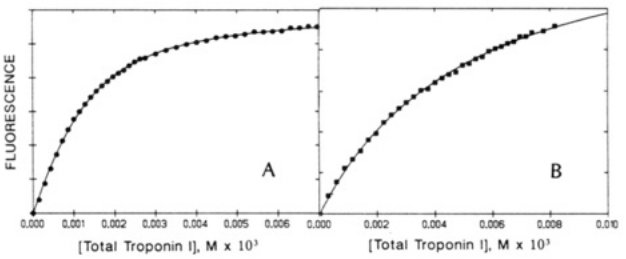

FIGURE 3: Titration of dansyl-troponin C fragments with troponin I. Conditions: 1 μM dansyl-troponin C fragment, 10 mM Pipes, pH 7.0, 0.3 M KCl, 3 mM DTT, and 0.01% NaN_3; T = 20 °C; λ_{ex} = 340 nm, λ_{em} 520 nm. Initial sample volume = 1.5 mL. [Stock troponin I titrant] = 44.25 μM. (A) Titration of dansyl-TnC-N-terminal fragment in the presence of 1 mM $CaCl_2$. (B) Titration of dansyl-C-terminal fragment in the presence of 3 mM $MgCl_2$ and 1 mM EGTA. Results are expressed as the volume-corrected fluorescence relative to the initial fluorescence. Data points represent typical single experiments.

A 15% gel of the purified fragments is shown in Figure 2. The purity of the fragments was estimated to be greater than 90% from the gels. This identification is consistent with their assignment to strong and weak calcium binding sites as determined in this study.

Results of preliminary actomyosin ATPase assays using troponin reconstituted with the troponin C fragments and troponin I and troponin T suggest that the N-terminal troponin C fragment, alone or combined with the C-terminal fragment, gives a small measure of calcium control. These are consistent with earlier studies (Grabarek et al., 1981) of the regulated actomyosin ATPase with proteolytic fragments of troponin C which suggested that residues 89–100 of skeletal troponin C constituted an important site of interaction with troponin I; our N-terminal fragment contains most of this region, residues 89–97.

Interaction of the Troponin C Fragments and Troponin I. For studies of complex formation between troponin I and the troponin C fragments, the dansyl-labeled troponin C fragments were titrated with unlabeled troponin I. The buffer contained 0.3 M salt to assure solubility of the troponin I. Complex formation led to enhancement of the fluorescence signal for all three buffer conditions. Typical experimental binding isotherms for the N- and C-terminal fragments are shown in Figure 3. The signal was fit to a model for a binary interaction (eq 1) using nonlinear least squares to determine the parameters: the association constant, the stoichiometry, and the maximum value of the fluorescence. The equilibrium constants for measurements at the three buffer conditions are presented in Table I. The stoichiometries ranged from 0.9 to 1.1; the correct stoichiometry for all these interactions is likely 1:1. For both fragments, the interaction is strongest in the presence of saturating concentrations of calcium and weakest when no divalent ions are present. These measurements were made in duplicate or triplicate with three different preparations of the fragments with reproducible results. The increase in the association constant observed for the fragments in calcium buffer versus magnesium buffer is much less than was observed for the interaction of troponin C and troponin I (Ingraham & Swenson, 1984; Wang & Cheung, 1985).

Table I: Troponin I Binding to Dansylated Troponin C Fragments

condition	K_a (M^{-1})[a] dansyl-N-terminal fragment	dansyl-C-terminal fragment
calcium	$(1.6 \pm 0.1) \times 10^6$	$(1.7 \pm 0.6) \times 10^6$
magnesium	$(6.1 \pm 1.4) \times 10^5$	$(3.9 \pm 2.2) \times 10^5$
EDTA	$(9.0 \pm 2.6) \times 10^4$	$(2.0 \pm 0.2) \times 10^5$

[a] K_a values represent averages, ±SD, of fit parameters from three to six data sets. Experimental conditions and details of fitting are described under Materials and Methods.

Table II: Troponin I Inhibitory Peptide Binding to Troponin C and Its Fragments

condition	K_a (M^{-1})[a] troponin C	N-terminal fragment	C-terminal fragment
calcium	$(5.3 \pm 1.1) \times 10^5$	$(2.5 \pm 0.5) \times 10^5$	$(6.5 \pm 2.0) \times 10^4$
magnesium	$(6.4 \pm 2.2) \times 10^4$	$(7.7 \pm 0.1) \times 10^4$	$(3.0 \pm 1.8) \times 10^4$[b]
EDTA	$(9.5 \pm 4.2) \times 10^4$	$(7.5 \pm 1.7) \times 10^4$	nm[b]

[a] K_a values represent averages, ±SD, of fit parameters from three to six data sets. Experimental conditions and details of fitting are described under Materials and Methods. [b] Binding to this fragment was very weak under these conditions; nm, not measurable.

Interaction of Troponin C and Fragments with the Troponin I Peptide. To determine where the troponin I inhibitory peptide region of troponin I interacts with troponin C, its binding to the troponin C fragments and troponin C was studied in the presence of calcium and of magnesium and in the absence of divalent metal ions. As troponin C contains no tryptophan, complex formation was followed by the increase in fluorescence of the tryptophan residue added to the synthesized inhibitory peptide at the N-terminus. This was used rather than a large extrinsic probe to minimize probe effects on the peptide structure and on the interaction with the small troponin C fragments. Concentration ranges for the experiments were chosen to give high precision for determination of the equilibrium constant or the stoichiometry. Typical binding isotherms for troponin C and its fragments are presented in Figure 4. The association constants obtained from fitting the binding isotherms to eq 1 are presented in Table II. In all cases, the interactions were strongest in the presence of calcium and weakest in the absence of divalent metal ions. The stoichiometry was in all cases close to 1:1. For the cases without divalent metal ion, the interaction was weak, and shortage of the fragments precluded use of the

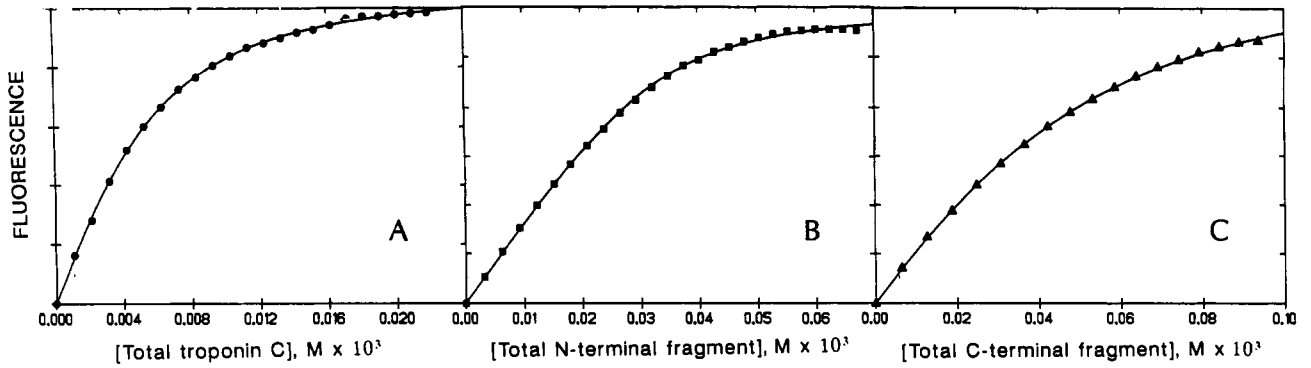

FIGURE 4: Fluorescence titrations of tryptophan-labeled troponin I inhibitory peptide with troponin C and troponin C fragments. Conditions: 10 mM Pipes, pH 7.0, 0.1 M KCl, 1 mM $CaCl_2$, 3 mM DTT, and 0.01% NaN_3; λ_{ex} = 295 nm, λ_{em} 350 nm. Initial sample volumes = 1.5 mL. (A) Troponin C titrant: initial [TnI inhibitory peptide] = 4.84 μM; [stock troponin C] = 320.6 μM. (B) Troponin C N-terminal fragment titrant: initial [TnI inhibitory peptide] = 38.68 μM; [stock N-terminal fragment] = 470.2 μM. (C) Troponin C C-terminal fragment titrant: initial [TnI inhibitory peptide] = 40 μM; [stock C-terminal fragment] = 644.7 μM. Results are expressed as the volume-corrected fluorescence relative to the initial fluorescence. Data points represent typical single experiments.

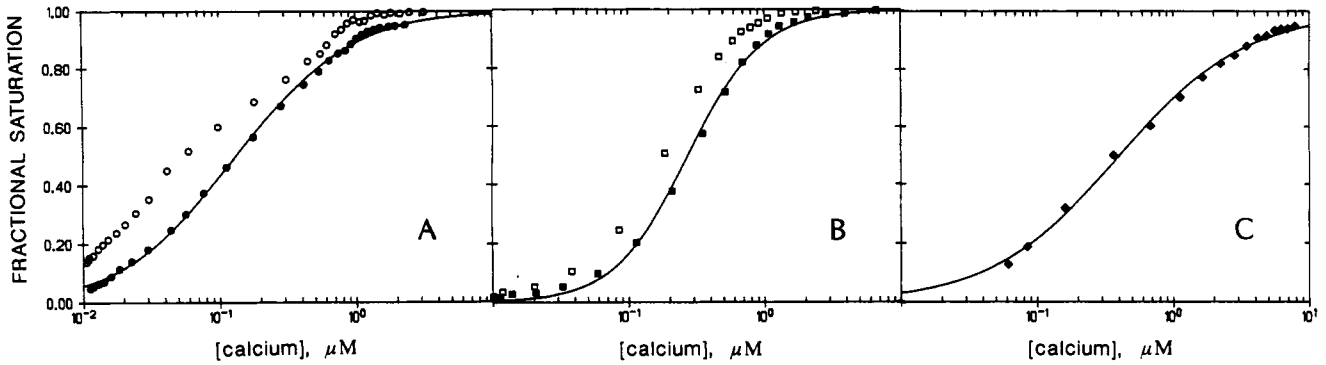

FIGURE 5: Calcium binding to troponin C and troponin C fragments: effects of troponin I inhibitory peptide binding. Conditions: 5 μM protein, 5 μM calcium fluorophore, 20 mM Tris, pH 7.5, 0.1 M KCl, 1 mM DTT, and 0.01% NaN_3. Initial total calcium concentrations for all samples were sufficient to ensure >99% saturation of all calcium binding species. Changes in BAPTA (for troponin C and C-terminal fragment titrations) or 4,4′-difluoro-BAPTA (for the N-terminal fragment) and tyrosine fluorescence were used to calculate free calcium concentrations and fractional saturations, respectively (see Figure 6). (A) (●) Troponin C, (○) troponin C + 56 μM troponin I inhibitory peptide; (B) (■) troponin C C-terminal fragment, (□) troponin C C-terminal fragment + 56 μM troponin I inhibitory peptide; (C) troponin C N-terminal fragment.

10–50-fold higher concentrations required to rigorously determine the stoichiometry. For these cases, the stoichiometry was fixed at 1:1 for determination of the association constants.

Our association constant for the interaction of the inhibitory peptide with troponin C in the presence of calcium ion (Table II) is slightly larger than the range, (7–10) × 10^4 M^{-1}, reported by Lan et al. (1989) using the non-covalently-bound probe 6-(*p*-toluidino)naphthalene-2-sulfonate. Although association constants for troponin C fragments were not measured in this study, those for the N- and C-terminal tryptic fragments of calmodulin were 7 × 10^3 and 8 × 10^4 M^{-1}, respectively. The association constant for the C-terminal fragment is in good agreement with what we observe, but that for the N-terminal fragment is significantly smaller. As calmodulin was fragmented at a different point in the interconnecting helix and interaction was detected with a different probe, further comparison seems presumptuous. It is notable that the stoichiometry consistent with all these measurements was also 1:1.

Binding of Calcium to the Troponin C Fragments. Calcium binding to the troponin C fragments was investigated to determine (1) if cleavage at residue 97 had any effect on the conformations of the two calcium binding domains and (2) if saturating concentrations of the troponin I inhibitory peptide affected calcium binding of the fragments, i.e., an indirect measure of interaction of the troponin I inhibitory peptide with the troponin C fragments and troponin C.

Typical calcium binding curves for each of the troponin C fragments and troponin C are presented in Figure 5. For calcium binding to troponin C, the N-terminal and C-terminal

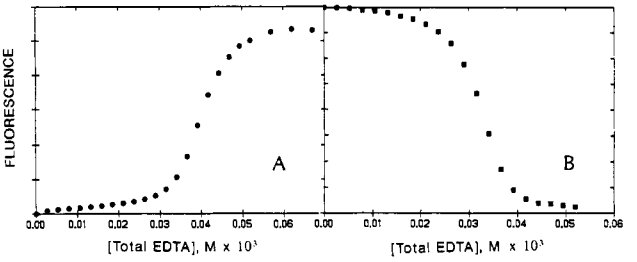

FIGURE 6: Typical raw data curves for dependence of BAPTA and tyrosine fluorescence on free calcium concentration. See Figure 5 legend for experimental conditions. Fractional saturations were calculated after each addition using volume-corrected tyrosine fluorescence measurements and corresponding limiting values of tyrosine fluorescence. Free calcium concentrations were calculated using eq 3. Results are expressed as the volume-corrected fluorescence relative to the initial fluorescence. (A) 5 μM BAPTA; (B) 5 μM troponin C C-terminal fragment tyrosine.

fragments contributed, respectively, ~15% and ~85% of the tyrosine signal enhancement. These experiments were also performed in the presence of troponin I inhibitory peptide at a concentration sufficient to achieve >90% saturation. In Figure 6 are the tyrosine and BAPTA fluorescence data as a function of added EDTA from which the binding curves were calculated using eq 3.

The association constants for calcium binding to the two fragments, determined as parameters of the fit of the data to eq 4, are presented in Table III. Use of intrinsic tyrosine fluorescence, with its small signal for calcium binding to the

Table III: Calcium Binding to Troponin C and Troponin C Fragments ±Troponin I Inhibitory Peptide

calcium binding protein	K_a (M^{-1})[a] −troponin I peptide	% change[b] +troponin I peptide
troponin C (strong sites)	$(1.0 \pm 0.1) \times 10^7$	+152
N-terminal fragment	$(2.8 \pm 0.2) \times 10^6$	nm[c]
C-terminal fragment	$(3.7 \pm 0.4) \times 10^6$	+70

[a] K_a values represent averages, ±SD, of fit parameters from three to six data sets. Experimental conditions and details of fitting are described under Materials and Methods. [b] The affinity of the troponin I inhibitior peptide for these calcium binding proteins decreases as the titration proceeds. Therefore, values for K_a percent change, calculated as $100\{[K_a(+\text{troponin I peptide}) - K_a(-\text{troponin I peptide}]/K_a(-\text{troponin I peptide})\}$, represent a lower limit to the changes in calcium affinity induced by TnI inhibitory peptide binding. [c] Reproducible results were not possible due to experimental difficulties.

N-terminal domain relative to the C-terminal domain, precluded determination of the association constants for the weak sites in troponin C. The association constants are of the same magnitude as those determined for troponin C and similar troponin C fragments (Potter & Gergely, 1975; Leavis et al., 1978). The data do suggest that the weak sites bind calcium more tightly in the N-terminal fragment than in troponin C. The Hill coefficients for the N-terminal fragment and both classes of troponin C sites were 1–1.2 while for the C-terminal fragment it was ~1.7. Calcium binding appears to be cooperative to the C-terminal fragment (Grabarek et al., 1983).

In the presence of near-saturating concentrations of troponin I inhibitory peptide, the calcium association constants of the strong sites of troponin C and the C-terminal fragment were increased 2.5-fold (pCa increase of 0.4). For the N-terminal fragment alone, the data (not shown) suggested that the strength of calcium binding was not affected. The data from this experiment were not reproducible. The contribution of the weak sites to the signal for calcium binding to troponin C is small, but in that case, it appeared that the bound inhibitory peptide strengthened calcium binding to the weak sites as well. This is in agreement with our direct binding studies.

As the ionic strength used in our calcium binding studies, ~0.11, is different from earlier measurements on BAPTA (Tsien, 1980) and 4,4′-difluoro-BAPTA (Smith et al., 1983), we measured their dissociation constants using the calcium buffer system EGTA/NTA. In a fluorescence titration, the extent of complexation of BAPTA or 4,4′-difluoro-BAPTA with calcium was measured, and the total calcium concentration was used to calculate the free calcium concentration. A typical binding isotherm fitted to eq 5 is presented in Figure 7. The dissociation constants for BAPTA and 4,4′-difluoro-BAPTA so determined are 4.12×10^{-8} and 2.67×10^{-7} M, respectively. They were used in the calcium binding studies on troponin C and the fragments.

Discussion

The calcium-specific and the calcium-magnesium domains and the interconnecting helix of troponin C participate in calcium-dependent interactions with troponin I which are part of the calcium-sensitive switch of skeletal muscle. At least three sites of interaction have been implicated. We have dissected this interaction by performing direct binding studies using fragments of troponin C and the troponin I inhibitory peptide. For the troponin C fragments, we have also measured their calcium binding properties. These measurements provide information on structural changes induced in the calcium binding domains upon cleavage and on the interactions that may be part of calcium control. We will first discuss the calcium binding studies and then studies of the direct binding

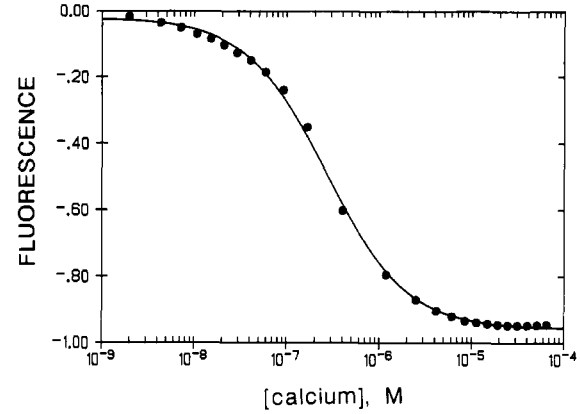

FIGURE 7: K_d determination of calcium fluorophore. A typical calcium binding curve for 4,4′-difluoro-BAPTA is shown. $K_d = 2.67 \times 10^{-7}$ M^{-1} as determined by the fit of the data to eq 5.

interactions between troponin C and its fragments and troponin I and the troponin I inhibitory peptide.

The results of our calcium binding studies show that the affinities of the strong and weak sites in the fragments are different than in troponin C. The association constant of the strong sites within the C-terminal fragment is approximately 2.5-fold lower than for troponin C. This is not unexpected as the TNB-CN cleavage site is just five residues to the amino-terminal side of the site III calcium binding loop, thus likely affecting the N-helix of this particular EF-hand calcium binding motif which is known to affect the binding properties (Shaw et al., 1991). Interestingly, the calcium affinity of the N-terminal fragment (weak sites) is significantly increased as compared to its affinity in troponin C (Zot & Potter, 1984; Potter & Gergely, 1975). This suggests that in troponin C the calcium-saturated strong sites decrease the calcium affinity of the weak sites relative to the N-terminal fragment. This domain–domain interaction, which can be characterized as a positive free energy of interaction (Weber, 1975), is absent in the fragments. The calorimetric studies of Tsalkova and Privalov (1985) on troponin C noted domain–domain interaction in troponin C.

For troponin C, the fitted value of K_a for the strong sites using our method was slightly smaller but in good agreement with those reported (Potter & Gergely, 1975; Johnson et al., 1978). The small fluorescence signal from saturation of the weak sites relative to the strong sites in troponin C precluded definitive evaluation of their K_a for calcium.

Calcium binding to the C-terminal fragment (strong sites) and troponin C is increased in the presence of near-saturating levels of the troponin I inhibitory peptide. The K_a increases by a factor of 2–3. Similar results were obtained with the N-terminal fragment, but the precision of the data is lower. Van Eyk et al. (1991) noted an increase in the strength of calcium binding to the weak sites in skeletal troponin C in the presence of the inhibitory peptide; they did not, however, observe an increase in the strength of calcium binding to the strong sites in troponin C in the presence of the inhibitory peptide. Our indirect measurements of peptide binding are in good agreement with the direct measurements discussed below as is required by microscopic reversibility. Calcium binds more strongly to the troponin C fragments when peptide is bound; thus, the troponin I inhibitory peptide binds more strongly to the troponin C fragments when they are saturated with calcium.

Both troponin C fragments bind to troponin I with similar association constants and 1:1 stoichiometry. Earlier studies have suggested an interaction between residues 1–21 of tro-

ponin I (Weeks & Perry, 1978) and the N-terminal calcium binding domain and between the troponin I inhibitory peptide and residues 89–100 of troponin C (Zot & Potter, 1987). It might be assumed that the fragments bind to the same sites on troponin I as they do when they are part of the troponin C molecule; our results of course give no information on the location of the binding sites but are consistent with this assumption. Specifically, the association constant for the C-terminal fragment, which corresponds to the calcium–magnesium domain of troponin C, is 4-fold larger when calcium rather than magnesium ions are bound to the sites. The association constant for the N-terminal fragment, which corresponds to the calcium-specific domain and contains two of the three proposed sites of interaction for troponin I, is larger by a factor of 3 with bound calcium versus magnesium ions. These increases are the same within experimental error. In the absence of divalent metal ions, the association constants for the N- and C-terminal fragments are smaller than those for calcium by factors of 17 and 8, respectively. To a first approximation, one might expect additivity of the interactions in the troponin C–troponin I complex which suggests that the product of the association constants for the fragments would equal the association constant of the troponin C–troponin I complex. However, the products of fragment association constants, which range from 10^{12} in the presence of calcium ion to 10^{10} in the absence of divalent ions, are much larger than the constants for the troponin C–troponin I interaction which range from 10^{9} in the presence of calcium to 10^{6} in the absence of divalent metal ions (Ingraham & Swenson, 1984; Cheung et al., 1987). Correction for the cratic free energy component would make the discrepancy larger in all cases.

These data suggest a substantial positive free energy of interaction in the binding of troponin C to troponin I relative to the fragments binding to troponin I; the average strength of the interactions is larger with the fragments than with troponin C. Also, the calcium dependence of the strength of the interactions is much smaller with the fragments than with troponin C. The relationship between these two observations is imperative, but it is not obvious. The calcium dependence must arise from the structural features of the troponin C–troponin I interface. It has been shown that a mutant troponin C with a disulfide linkage in the N-terminal domain which precludes movement of one of the helical segments removes calcium sensitivity and abolishes binding to troponin I (Grabarek et al., 1990; Gusev et al., 1991). Thus, one contribution to the large calcium dependence of the interaction between troponin C and troponin I may be relief of the domain–domain interaction in troponin C. This can be done by a calcium-dependent conformational change in the N-terminal domain which strengthens the interactions with troponin I. Relative to troponin C, however, the fragments, lacking the domain–domain interaction, can bind to troponin I quite well without a conformational change in the N-terminal fragment as calcium binding only increases the association constant 2-fold.

Several aspects of the results on the binding of the troponin I inhibitory peptide to troponin C and its fragments are of interest. There is an approximate 8-fold increase in K_a in the presence of bound calcium versus magnesium ion for binding to troponin C (Table II). A smaller 3-fold increase in K_a is noted for the N-terminal fragment. No difference was detected for the C-terminal fragment within experimental error. As was observed for the binding of the troponin C fragments to troponin I, the product of the binding constants for the troponin I inhibitory peptide to the TnC fragments ($\sim 10^{10}$ in calcium buffer, $\sim 10^{9}$ in magnesium buffer) is much greater than the binding constant for troponin C (see Table II). Analogous to the binding of the troponin C fragments to troponin I, these observations reflect a significant positive free energy of interaction for the troponin I inhibitory peptide interaction with troponin C relative to the troponin C fragments.

These results are difficult to understand in terms of a binding site on troponin C for the troponin I inhibitory peptide region of troponin I which includes only residues 89–100. If binding is to this sequence, little calcium dependence of troponin I peptide binding would be expected. Given the results on deletion mutants which showed that residues 84–94 were not required for function, the troponin I inhibitory peptide is likely to have additional interactions with the globular part of the N-terminal domain. Recent cross-linking studies have suggested the feasibility of such an interaction (Leszyk et al., 1990). It is surprising that the C-terminal fragment of troponin C, possessing only the last three residues of the troponin C critical site, binds the troponin I inhibitory peptide at all. A calcium-independent interaction with troponin I has been suggested for this domain (Grabarek et al., 1981) but not necessarily with the troponin I inhibitory peptide. A final interesting and confusing point is that the stoichiometry for all the interactions is 1:1. Troponin C, which contains both of the fragments, binds only one molecule of inhibitory peptide in spite of substantial interaction of the peptide with the individual fragments; clearly, additivity of the interaction strengths does not apply.

Some insight into these observations is possible given features of the troponin C and troponin I inhibitory peptide structures. Observations on the crystal structure have shown that each globular domain contains a hydrophobic "cup"—a site dominated by hydrophobic side chains (Strynadka & James, 1988). Numerous solution studies have shown that phenathiazine drugs bind to these hydrophobic regions [see, e.g., Massom et al. (1990)]. A recent NMR solution structure of the troponin I inhibitory peptide bound to troponin C has shown that this highly positively charged sequence, GKFKRPPLRRVR, is bent in two places to form a structure with an exposed hydrophobic core and a positively charged edge (Campbell & Sykes, 1989). The binding free energy for the interaction of this peptide with troponin C and its fragments can have hydrophobic and electrostatic contributions. Solution structures for troponin C which are considerably more compact than the crystal structure have been suggested by several studies. These include fluorescence distance measurements and low-angle X-ray and cross-linking studies (Cheung & Wang, 1989; Heidorn & Trewhella, 1988; Leszyk et al., 1990; Kobayashi et al., 1991). These latter studies have shown that an interaction is feasible between the troponin I inhibitory peptide and the N-terminal domain of troponin C. The most recently reported cross-linking studies found a link between Cys-57 (mutant troponin C) and residues in the troponin I inhibitory peptide (Kobayashi et al., 1991). The results led these authors to suggest a more compact structure for troponin C. Obviously, our experiments do not indicate the actual sites of interaction between troponin C and its fragments and the troponin I inhibitory peptide and troponin I, nor do they suggest structure. Our results do show that the troponin I inhibitory peptide interacts with both troponin C domains; only the N-terminal domain shows significant calcium dependence of binding. Direct involvement of the N-terminal domain in conformational switching is more attractive than involvement of the C-terminal domain. The latter is saturated with magnesium throughout the contraction cycle.

Our results are more readily understood if the N- and C-terminal domains of troponin C are close enough to form a single binding site for the troponin I inhibitory peptide within a compact structure. The interaction would contain both electrostatic and hydrophobic components, and the site would include portions of both domains of troponin C with small contributions from the interconnecting helix. Part of the physical basis for the calcium switch might then involve a calcium-dependent conformational change in the N-terminal fragment which is coupled to decreases in an unfavorable domain–domain interaction in troponin C.

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Registry No. Ca^{2+}, 7440-70-2; ATPase, 9000-83-3; Mg^{2+}, 7439-95-4.

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Role of Protein–Protein Interactions in the Regulation of Transcription by *trp* Repressor Investigated by Fluorescence Spectroscopy†

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ABSTRACT: In the present work, we have characterized the protein–protein interactions in the *trp* repressor (TR) from *Escherichia coli* using fluorescence spectroscopy. The steady-state and time-resolved fluorescence anisotropy of repressor labeled with 5-(dimethylamino)naphthalene-1-sulfonamide (DNS) was used to monitor subunit equilibria in the absence and presence of corepressor. In the absence of tryptophan, the repressor is in equilibrium between tetramers and dimers in the concentration range studied (approximately 0.04–40 μM in dimer). Binding of corepressor resulted in a marked destabilization of the tetramer. The beginning of a dimer–monomer dissociation transition was observed by monitoring the decrease in the intrinsic tryptophan emission energy upon dilution below 0.1 μM in dimer, indicating an upper limit for the dimer-dissociation constant near 1 nM. DNA titrations with a 26 base pair sequence containing the *trp* EDCBA operator performed in the absence and presence of the corepressor are consistent with a 1:1 dimer/operator stoichiometry in the presence of tryptophan, while the aporepressor binds with TR dimer/DNA stoichiometries greater than one and which depend upon both the concentration of protein and that of the operator. Using the multiple observable parameters available in fluorescence, we have thus carried out a thorough investigation of the coupled equilibria in this bacterial repressor. Our results are consistent with a physiologically relevant thermodynamic role for tetramerization in the regulatory function of the *trp* repressor. The present results which have brought to light novel protein–protein interactions in the *trp* repressor system indicate that fluorescence spectroscopic methods could prove quite useful in the study of the role of protein–protein interactions in eukaryotic systems as well.

Protein–protein interactions have emerged as one of the underlying general mechanisms governing the regulation of transcription. Such protein oligomerization interactions intervene in regulating the binding to DNA by prokaryotic repressors such as the *arc* (Bowie & Sauer, 1989), *lambda* (Senear & Ackers, 1990), and *lac* repressors (Royer et al., 1990). Differential dimerization affinities are involved in the activation of eukaryotic transcription by the oncogene products fos and jun (Turner & Tijan, 1989; Gentz et al., 1989; Kouzerides & Ziff, 1989), which also play a role through protein–protein interactions in modulating the activity of hormonal receptors (Diamond et al., 1990). Both positive and negative regulating dimerization partners have been identified for a number of helix–loop–helix-type proteins implicated in development and differentiation (Baringa, 1991; Blackwood & Eisenman, 1991; Prendergast et al., 1991). The three-dimensional structure for several DNA-binding proteins, both alone and complexed with their cognate DNA sequences, have been solved, including those of the *trp* repressor (Schevitz et al., 1985; Zhang et al., 1987; Otwinowski et al., 1988), and these provide the framework for an understanding of their function. However, a complete understanding of the physi-

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