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REVIEW

Molecular mechanism of troponin-C function

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Introduction

The thin filament of striated (skeletal and cardiac) muscle is composed of actin, tropomyosin and troponin (Tn), the last two proteins being essential for Ca^{2+} regulation of contraction (Ebashi & Endo, 1969). Troponin is a complex of three proteins: troponin C (TnC), the Ca^{2+}-binding component; troponin-T (TnT), the tropomyosin binding subunit; and troponin I (TnI), the inhibitory subunit, so-called because it is capable of binding to actin and inhibiting actomyosin ATPase activity (Schaub & Perry, 1969; Greaser & Gergely, 1971). Calcium released on neural stimulation from the sarcoplasmic reticulum binds to TnC, triggering a sequence of events that ultimately give rise to contraction. Early structural studies provided the basis for a model of thin filament regulation known as the 'steric blocking model' (Haselgrove, 1972; Huxley, 1972; Parry & Squire, 1973). According to this model in the relaxed (Ca^{2+}-free) state of muscle tropomyosin assumes a position on the actin filament in which it effectively blocks the attachment of myosin crossbridges to actin. On activation, tropomyosin moves to clear the myosin–actin interaction site, thereby permitting crossbridge cycling and contraction of the muscle. Recent time-resolved X-ray diffraction studies on muscle fibres show some mass redistribution preceding crossbridge attachment that may be interpreted as tropomyosin movement (Kress *et al.*, 1986) but no unequivocal evidence of such movement has to date been obtained. Moreover, in the presence of MgATP, Ca^{2+} only slightly enhances the binding of myosin heads to regulated actin (containing tropomyosin and troponin) and to an extent that would not account for the enhanced ATPase activity. To explain these results it was proposed that in the relaxed state tropomyosin blocks not the myosin attachment step but one of the subsequent steps in the ATP-driven crossbridge cycle (Chalovich & Eisenberg, 1982). Whether or not tropomyosin moves the activation appears to involve Ca^{2+}-dependent dissociation of TnI from actin which releases tropomyosin from its inhibitory position or state. Recently, direct evidence for the movement of TnI away from actin as part of the regulatory process has been provided by resonance energy transfer and photocrosslinking studies (Tao *et al.*, 1990).

Various aspects of regulation of contraction have been previously reviewed (Eisenberg & Hill, 1985; Leavis & Gergely, 1984; El-Saleh *et al.*, 1986; Ohtsuki *et al.*, 1986; Phillips *et al.*, 1986; Zot & Potter, 1987; da Silva & Reinach, 1990; Strynadka & James, 1991). In this review we summarize data on troponin C, especially those based on X-ray crystallography and site-directed mutagenesis, and relate them to the mechanism of thin filament-based regulation in striated muscle. In our discussion we assume that the conclusions reached for TnC from avian (turkey and chicken) skeletal muscle are directly applicable to rabbit skeletal TnC, the protein that has been studied most extensively. Where appropriate we also refer to cardiac TnC and to calmodulin, an ubiquitous Ca^{2+} regulatory protein homologous to TnC.

Troponin C, a four-site, two-domain protein

Troponin C is an 18 kDa acidic protein that belongs to the family of proteins utilizing a common motif for binding Ca^{2+}, a 12-residue loop flanked by two α-helical segments. This helix-loop-helix (HLH) unit, also called EF-hand, was first described by Kretsinger and Nockolds (1973) based on X-ray diffraction data on crystals of parvalbumin. The loop and a residue in the C-terminally adjacent helix provide seven oxygen ligands resulting in pentagonal bipyramidal Ca^{2+}-coordination (for reviews see Kretsinger, 1980, 1987; Strynadka & James, 1989). In the amino acid sequence of TnC from rabbit skeletal

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muscle there are four homologous HLH motifs (Collins *et al.*, 1977) corresponding to its four metal binding sites (Potter & Gergely, 1975). The so-called low affinity sites bind Ca^{2+} specifically ($K = 3 \times 10^5$ M^{-1}) whereas the high affinity sites bind both Ca^{2+} and Mg^{2+} ($K_{Ca} = 2 \times 10^7$ M^{-1}, $K_{Mg} = 2 \times 10^3$ M^{-1}). The eight helical segments adjacent to the loops are labelled A–H starting from the N-terminus.

In the first attempt to predict the three-dimensional structure of TnC, Kretsinger and Barry (1975) assumed that each of the four helix-loop-helix units has a conformation essentially identical to that of the EF region of parvalbumin (Kretsinger & Nockolds, 1973) and that the units are arranged in two pairs, I–II and III–IV, with strong interactions within each pair. Kretsinger and Barry also assumed, by analogy to parvalbumin, a compact arrangement of the two pairs, the side chains of the helical segments forming the hydrophobic core of the molecule. The recently obtained high resolution crystal structure of TnC (Fig. 1) from turkey (Herzberg & James, 1985, 1988) and chicken (Sundaralingam *et al.*, 1985a; Satyshur *et al.*, 1988) skeletal muscle is in agreement with several of the earlier predictions (for detailed review see Strynadka & James, 1989) but showed no evidence of contacts between the two pairs of sites. Instead the molecule has a considerably elongated dumbbell shape with the two globular domains connected by a single 31-residue-long central α-helix comprising helical segments D and E including the linker which had previously been considered non-helical. The two HLH units in each domain are tightly coupled via a short segment of β-sheet structure formed by the two Ca^{2+}-binding loops and numerous interhelical hydrophobic contacts, in good agreement with observations based on ^{1}H-NMR (Evans *et al.*, 1980; Drabikowski *et al.*, 1985). These contacts may serve as a source of cooperativity of Ca^{2+}-binding within each domain. In the crystals Ca^{2+} is bound only at the high affinity sites III and IV whereas the low affinity sites I and II are Ca^{2+}-free. This feature eventually proved crucial for the development of a model of the Ca^{2+}-induced conformational transition in TnC (Fig. 2).

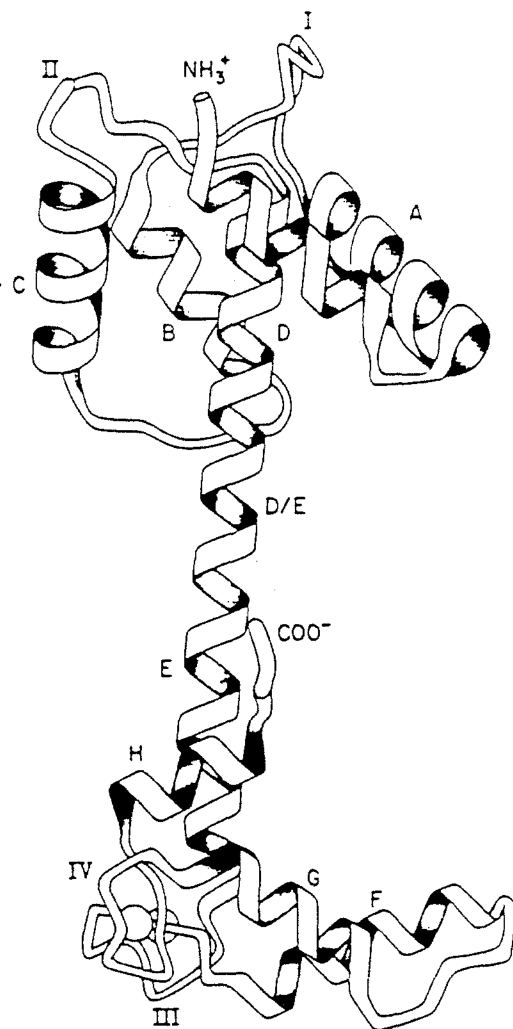

Fig. 1. Ribbon model derived from X-ray diffraction of skeletal muscle TnC crystals. Helices are labelled A–H; Ca^{2+}-binding sites are labelled I–IV. From Herzberg and James, 1985; by permission.

Crystal versus solution structure of TnC

The single central α-helix in TnC is a rather unusual feature and may be expected to be unstable owing to its exposure to the solvent and the presence of a Gly residue, a typical helix breaker, in its middle. Bending or local unfolding of the central helix would significantly alter the global structure of the molecule by changing the relative orientation and/or distance between the two domains, and could have an effect on the physiological properties of TnC. It has been suggested that the helical structure of the central helix may be stabilized by a number of ionic interactions between the side chains of Lys or Arg and Glu separated by three residues (approximately one helical turn) (Sundaralingam *et al.*, 1985b). Although such an effect has been observed in synthetic peptides (Marqusee & Baldwin, 1987) the atomic structure of TnC provides little evidence for such interactions (Strynadka & James, 1989). The question remains whether the central helix is stable enough to maintain the extended structure of TnC in solution or whether such a structure exists only in the crystal latice owing to intermolecular interactions. It is also not clear what effects the other troponin components may have. Evidence accumulated over the years indicate both structural independence of the two domains and some interdomain interactions.

Structurally independent domains

Perhaps one of the earliest indications of the domain structure of TnC came from studies on limited proteolysis. In its Ca^{2+}-bound form TnC can be cleaved with trypsin into two spontaneously separating fragments (TR_1C and TR_2C) of nearly equal size (Drabikowski *et al.*, 1976; Grabarek *et al.*, 1981b). Both fragments preserve their original Ca^{2+}-binding properties (Leavis *et al.*, 1978)

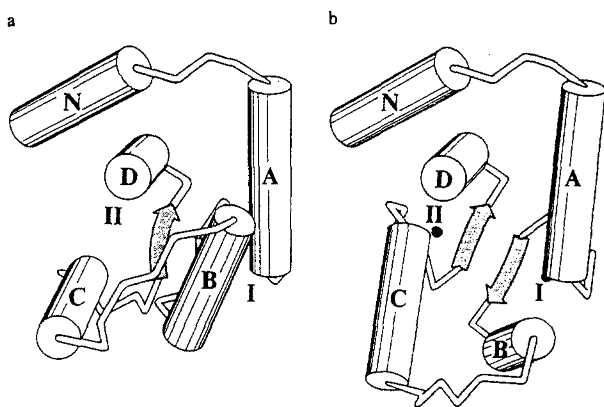

Fig. 2. Schematic representation of the Ca^{2+}-induced triggering conformational transition in the N-terminal domain of TnC (from Strynadka & James, 1989, by permission). (a) the Ca^{2+}-free crystallographic structure; (b) the predicted Ca^{2+}-bound conformation. In this model helices N, A and D retain their relative disposition. Helices B and C and the linker peptide move when Ca^{2+} binds. The hydrophobic cavity exposed in the Ca^{2+}-bound form provides an interaction site with TnI. A similar site is located in the C-terminal domain.

and their structural integrity, as evidenced by circular dichroism (Leavis *et al.*, 1978) and ^{1}H-NMR (Evans *et al.*, 1980; Drabikowski *et al.*, 1985). Microcalorimetric studies of Tsalkova and Privalov (1980) clearly indicated two separate cooperative units in TnC corresponding to the two classes of Ca^{2+}-binding sites. The stability of each of the two-site domain in isolated form is similar to that in the native molecule (Brzeska *et al.*, 1983; Tsalkova & Privalov, 1985). In contrast, single-site-peptides obtained by proteolysis or chemical synthesis have much lower affinity for Ca^{2+} and are significantly less stable (Leavis *et al.*, 1978; Nagy *et al.*, 1978; Reid *et al.*, 1981; Leavis *et al.*, 1982), moreover their Ca^{2+}-binding ability appears to be attributable, at least in part, to dimerization (Shaw *et al.*, 1990, 1991; Kay *et al.*, 1991). These results indicate that a two-site unit represents a structural domain and that there is little interaction between the two domains in TnC, clearly in agreement with the crystallographic extended structure.

Interdomain interactions

On the other hand there are also data indicating some sort of interdomain communication in TnC. Wang and colleagues (1983) have shown that the dissociation rate of Ca^{2+} from sites III and IV in TnC is approximately three times slower when La^{3+} is bound at sites I and II. The fluorescent probe dansylaziridine attached to Met-25 in site I exhibits a large fluorescence increase associated with Ca^{2+} binding to sites I and II and also a small decrease in fluorescence in the $[Ca^{2+}]$ range corresponding to binding to sites III and IV (Johnson *et al.*, 1978). Conversely, whereas probes attached to Cys-98 in HLH unit III are primarily reporters of the conformation of sites III and IV they also show responses to Ca^{2+}-binding at sites I and II (Potter *et al.*, 1976; Grabarek *et al.*, 1986).

The interdomain communication is evident not only in the protein conformers present under equilibrium conditions but also in those that appear transiently on the time scale comparable to that of the activation process in muscle. Rosenfeld and Taylor (1985) have shown with the use of a fluorescent probe attached to Cys-98 that Ca^{2+} binding to the isolated C-terminal peptide containing only site III and IV (TR_2C) follows rather simple kinetics, whereas the same probe attached to intact TnC behaves in a more complicated way indicating that it is affected by Ca^{2+}-binding to sites I and II. Grabarek and colleagues (1986) have shown that a fluorescent probe at Cys-98 exhibits a rapid ($k > 150\ s^{-1}$) fluorescence increase on addition of Ca^{2+} to Mg^{2+}-saturated TnC. As in these experiments the rate of Ca^{2+}-binding to sites III and IV was limited by the slow ($k \sim 8\ s^{-1}$) Mg^{2+}-dissociation, the rapid change in fluorescence must be from Ca^{2+}-binding at the distant sites I and II. The effect was significantly enhanced in the complex with TnI.

Shape of the molecule: interdomain separation

The observations indicating interdomain interactions, discussed above, could easily be explained if the two domains in TnC could, at least transiently, come in contact. Several observations suggest that this indeed may be the case. Energy transfer studies employing lanthanides have shown that the distance between the Ca^{2+}-binding sites is consistent with the X-ray structure at low pH, but decreases substantially at neutral pH (Wang *et al.*, 1987a,b). The Ca^{2+}- and TnI-dependent changes in resonance energy transfer efficiency between two labels, each located in different domains, also indicate that the distance between the two domains is not fixed and depends on solution conditions and the occupancy of the cation binding sites (Cheung *et al.*, 1982, 1991; Wang & Cheung, 1984; Wang *et al.*, 1987a). Results of low angle X-ray scattering studies suggest that the mass distribution in TnC is consistent with a more compact molecule than that indicated by X-ray crystallography (Heidorn & Trewhella, 1988). Thus it appears that the extended structure revealed by X-ray crystallography is not the only structure TnC can assume and it is not clear what is the physiologically relevant structure.

Regions of functional significance in TnC

Ca^{2+}-binding sites

The question of the functional significance of each of the four Ca^{2+}-binding sites in TnC was first addressed by Potter and Gergely (1975). They found that in free TnC and in the whole troponin complex Mg^{2+} in the millimolar range decreases the apparent Ca^{2+}-binding constant to the high affinity sites but not that to the low affinity sites. As the ATPase activity of a regulated

reconstituted actomyosin, a complex containing myosin, F-actin, tropomyosin, TnI, TnT and TnC, as a function of [Ca^{2+}] was found not to depend on [Mg^{2+}] they concluded that the regulatory properties of TnC must be associated with the low affinity sites (Potter & Gergely, 1975), later identified as sites I and II (Leavis *et al.*, 1978; Sin *et al.*, 1978). More convincing were kinetic experiments (Johnson *et al.*, 1979; Iio & Kondo, 1980) supported by computer modelling (Robertson *et al.*, 1981). They showed that the dissociation of Mg^{2+} from the high affinity sites, which would have to precede Ca^{2+} binding at these sites if they were to be responsible for activation, was much too slow ($k_{off} = 8\ s^{-1}$) to account for the onset of tension in a living muscle (~ 15 ms).

Recent studies utilizing site directed mutagenesis cast more light on the nature of the regulatory site(s). With mutants of skeletal muscle TnC Sheng and colleagues (1990) found that inactivation of either of the low affinity sites by replacing Asp residues at coordinating positions 1 and 3 in the Ca^{2+}-binding loop with Val and Gly, respectively, significantly decreases the regulatory activity of TnC. This suggests that both low affinity sites are necessary for the activity. In the case of cardiac troponin C (cTnC), where owing to some amino acid substitutions site I does not bind Ca^{2+} (van Eerd & Takahashi, 1976; Burtnik & Kay, 1977; Leavis & Kraft, 1978; Holroyde *et al.*, 1980), site II has been regarded as that responsible for the regulatory function. Direct support for this view has been recently provided by the work on mutants of cTnC (Putkey *et al.*, 1989; Sweeney *et al.*, 1990). The cTnC mutant in which Ca^{2+}-binding to site I is restored but site II is deactivated was shown to be unable to regulate muscle fibres. Another mutant in which both sites I and II can bind Ca^{2+} showed higher cooperativity in the response of force to [Ca^{2+}] leading to the conclusion that site I in cTnC merely modulates the regulatory function of site II. It is not clear what causes the difference between TnC from cardiac and skeletal muscles.

The role of the high affinity sites III and IV is not entirely clear. It has been suggested that these sites play a structural role by providing stability to the troponin complex (Potter & Gergely, 1975; Zot & Potter, 1982). Recent results of Negele and colleagues (1992) have shown that the regulatory activity of cardiac TnC requires that at least one of these sites be able to bind Ca^{2+}. If both sites are inactivated the protein can regulate actomyosin ATPase only at much higher concentrations, the effect most likely being related to a significant decrease in affinity for TnI.

Central helix

The presence of the unusual single helix in TnC has spurred conjectures that it may be involved in the signal transduction process (Sundaralingam *et al.*, 1985b). To test this hypothesis Reinach and Karlsson (1988) used site-directed mutagenesis to replace a Gly in the middle of the central helix by Pro with the aim of disrupting the helix's integrity, however the replacement had no effect on the activity of TnC. Hitchcock-DeGregori and colleagues have tested effects of various deletions in the segment of the central helix of chicken skeletal TnC comprising the sequence KEDAKGKSEEE. They found that deletion of the tripeptide KGK or the heptapeptide KEDAKGK results in a slightly impaired inhibition of ATPase activity of regulated actomyosin in the absence of Ca^{2+} whereas deletion of EDA or KG from the same stretch causes a small decrease in the activation of actomyosin ATPase in the presence of Ca^{2+} (Xu & Hitchcock-DeGregori, 1988; Dobrowolski *et al.*, 1991a,b). Deletion of a tetrapeptide SEEE had no effect on the regulatory activity of TnC. The interpretation of these data is complicated by the observation that all the deletion mutants with the exception of the dKG mutant restored force and Ca^{2+} regulation to TnC-depleted rabbit skinned skeletal muscle fibres (Sheng *et al.*, 1991). These findings may be interpreted in two ways, either the distance between the two domains and their relative orientation – the parameters that must be altered in the deletion mutants if the central helix retains its helical conformation – are unimportant, or some distortions or unfolding of the central helix compensate for the possible effects caused by the deletions. In either case the central helix appears not to play a key role in the function of TnC.

Segments essential for Ca^{2+}-dependent regulation

Early studies aiming at closer identification of the segments of TnC involved in the activation process have indicated fragment CB9 (residues 84–135) as having a slight ability to activate reconstituted regulated actomyosin (Weeks & Perry, 1978). Studies on the proteolytic fragments of TnC have shown that the peptide containing the two low affinity sites (residues 9–84) does not activate actomyosin ATPase, however partial activity (approximately 30%) was found in a slightly larger peptide (residues 1–100) and in the peptide containing the C-terminal domain (residues 89–159). As the common segment for both active peptides was the stretch containing residues 89–100 corresponding to helix E it appeared that this segment may be involved in the activation process. A much higher activity (approximately 60%) was found with a peptide containing sites I–III (Grabarek *et al.*, 1981a). This peptide partially regulates (approximately 60%) force development when incorporated into TnC deficient fibres (Sheng *et al.*, 1989).

These results indicate that the interaction with both domains is necessary for the full regulatory activity of TnC. The same feature is more clearly evident in the case of the homologous Ca^{2+}-binding protein, calmodulin. In this case none of the single-domain peptides can activate the target enzymes (Newton *et al.*, 1984). In recent elegant studies utilizing calmodulin mutants, Persechini and Kretsinger (1988a) have shown that as long as the

two domains are joined by the polypeptide chain of the central helix, or by a genetically engineered interdomain disulfide link (even with the central helix cleaved) activity is preserved. They suggested that the central helix works merely as a flexible tether holding the two domains in proximity enabling their simultaneous interaction with a segment of the target protein (Persechini & Kretsinger, 1988b).

Interaction between TnC and TnI

It has become clear that the critical step in the regulatory process involves a change in the interaction between TnC and TnI and that this change is triggered by Ca^{2+}-binding to TnC. The equilibrium binding constants for the formation of the TnC–TnI complex reported by various authors differ significantly (Ingraham & Swenson, 1984; Leavis *et al.*, 1984; Wang & Cheung, 1985; Cheung *et al.*, 1987). It is clear, however, that Ca^{2+} causes a large increase in affinity for TnI; the equilibrium binding constant being approximately 10^6 M^{-1} and 10^9 M^{-1} in the absence and presence of Ca^{2+}, respectively. The Ca^{2+}-induced increase in the negative free energy of TnC–TnI binding is coupled to the TnI-induced increase in the affinity for Ca^{2+} in both domains of TnC (Potter & Gergely, 1975; Wang & Cheung, 1985; Cheung *et al.*, 1987).

Sites of interaction

A considerable effort has been put into characterization of the interface between TnC and TnI. Studies on CNBr fragments of TnI (Syska *et al.*, 1976) have identified two segments involved in the interaction: one at the N-terminus (CN5) comprising residues 1–21, the other (CN4) comprising residues 96–116. The latter has also been found to bind actin and to inhibit actomyosin ATPase. Studies on synthetic peptide analogues of CN4 narrowed down the minimum inhibitory stretch to residues 104–116 (Nozaki *et al.*, 1980; Talbot & Hodges, 1981; Katayama & Nozaki, 1982). ^{1}H-NMR studies pointed to several positively charged and hydrophobic residues in the N-terminal part of CN4 as possibly being involved in the interaction with TnC, whereas several Arg residues mostly in the C-terminal part of CN4 were implicated in the interaction with actin (Dalgarno *et al.*, 1982; Grand *et al.*, 1982). Replacement with Gly of any of the residues in the synthetic peptides corresponding to the stretch 104–116 of TnI has been shown to affect to various extents the interaction with TnC and the inhibitory properties of the peptide (van Eyk & Hodges, 1988). Much less is known about the functional significance of the N-terminal segment of TnI corresponding to the CNBr fragment CN5. ^{1}H-NMR data of Dalgarno and colleagues (1982) implicated residues 6–16 including several Arg side chains in the interaction with TnC. Recently Ngai and Hodges (1991) have shown that a synthetic peptide comprising residues 1–40 of TnI prevents TnC from reversing the inhibition by CN4 of actomyosin ATPase. These results suggest that the N-terminal segment of TnI may play a role in stabilizing the inhibited state of the thin filament.

The interaction interface of TnC was found to be more extensive involving segments from both domains. On the basis of studies on proteolytic fragments of TnC, Grabarek and colleagues (1981a) proposed that helical segments C, E and G, the N-terminal helices of the Ca^{2+}-binding sites II, III and IV, respectively, are involved in the interaction. The involvement of helix E could be easily tested owing to the presence of the readily modifiable Cys-98. TnC with a spin probe attached to Cys-98 was found to broaden ^{1}H-NMR signals of the CN4 peptide (Dalgarno *et al.*, 1982). A benzophenone photocrosslinker attached to Cys-98 readily crosslinks TnC to TnI via the CN4 region of TnI (Leszyk *et al.*, 1987, 1988). Both observations are consistent with the interaction between CN4 and the region adjacent to Cys-98 in TnC.

Surprisingly, in more recent studies using zero-length crosslinking, the same stretch of TnI has been found to crosslink to the C-helix in site II of TnC (Leszyk *et al.*, 1990). Also a benzophenone photocrosslinker attached to a Cys residue substituted for Ala57 in helix C of a TnC mutant was found to crosslink to the C-terminal part of the inhibitory segment of TnI (residues 113–121) (Wang *et al.*, 1990a; Kobayashi *et al.*, 1991).

In spite of a great deal of information on the interacting segments in the TnC-TnI complex it is still unclear what is the topography of the interaction interface and what are the forces responsible for the interaction. In the work on TnC fragments, Grabarek and colleagues (1981a) emphasized the role of charge-charge interactions between TnC and TnI and pointed out the similarity of the distribution of negatively-charged residues in helical segments of TnC. Subsequent work directed attention at the involvement of hydrophobic interactions. Using ^{1}H-NMR Cachia and colleagues (1983) have shown that a synthetic analogue of the inhibitory segment of TnI affects the hydrophobic site chains in the C-terminal domain, in particular in helix E. Also the exposure of hydrophobic sites in TnC on Ca^{2+}-binding resulting in an increased affinity for hydrophobic probes has been documented (for review see Cachia *et al.*, 1985). In their analysis of the sequences of 30 helix-loop-helix units, Gariepy and Hodges (1983) pointed out characteristic distribution of hydrophobic and charged residues and discussed their involvement in interactions with target sites. Both the positively-charged and hydrophobic side chains in the inhibitory segment of TnI are affected on interaction with TnC (Grand *et al.*, 1982). Thus it appears that both charge–charge interactions and hydrophobic interactions are involved in formation of the TnC–TnI complex. Perhaps the most detailed description of a putative binding site in a two-Ca^{2+}-binding site domain was given by Strynadka and James (1990) in their

molecular modeling studies. They pointed out that in the crystal packing the C-terminal domain of TnC interacts with helix A of another symmetry-related molecule and proposed that some features of this interaction may be applicable to the interaction between TnC and TnI in the C-terminal domain. According to Strynadka and James the binding site appears to involve all four helical segments associated with two Ca^{2+}-binding units. The hydrophobic contacts play a primary role but some of the negatively-charged groups surrounding the hydrophobic cleft also contribute to the binding.

Significance of the domain structure

Although at present the exact structure of the TnC–TnI complex is not known the results discussed above strongly suggest that both domains of TnC interact with the same relatively short segment of TnI corresponding to residues 96–116. The situation is reminiscent of the interaction between calmodulin and its target proteins as revealed by molecular modeling studies (Persechini & Kretsinger, 1988b). In these studies the interacting segment of the target protein, the so called M13 peptide of the myosin light chain kinase, is represented by an amphiphilic α-helix whose hydrophobic sides interact with hydrophobic pockets formed by four helical segments in each of the two domains of calmodulin. This requires a bend in the central helix such that the two domains face each other. The question arises as to what extent this model is applicable to the TnC–TnI interaction. The presence of the Pro–Pro sequence makes it unlikely that the inhibitory segment of TnI adopts the conformation of a continuous α-helix. In fact the ^{1}H-NMR data of Campbell and Sykes (1991) on the complex of TnC with the inhibitory segment of TnI suggest a hairpin-like structure composed of two helices connected by the two prolines. It remains to be shown whether this structure is representative of the intact TnI and its complex with TnC. Nevertheless, the concept derived from studies on calmodulin of a complex having a compact structure in which the central helix is bent or locally unfolded so that both domains of TnC can interact with the inhibitory segment of TnI should be given serious consideration.

Ca^{2+}-induced conformational changes in TnC

What are the conformational changes in TnC induced by Ca^{2+} and how do they affect TnC's ability to interact with TnI? These are key questions concerning the activation process. Early spectroscopic work has shown that Ca^{2+}-binding to TnC results in approximately 50% increase in α-helix content (Murray & Kay, 1972; van Eerd & Kawasaki, 1972). Subsequently this increase has been attributed almost entirely to the C-terminal domain (Johnson & Potter, 1978; Leavis *et al.*, 1978). According to UV absorbance and CD studies helices F and G remain folded in the absence of Ca^{2+} whereas helices E and H undergo coil-helix transition on Ca^{2+}-binding at sites III and IV (Nagy & Gergely, 1979). These observations provided little insight into the regulatory process because, as discussed above, sites III and IV are believed to be always saturated in the resting state and the slow dissociation rates exclude them from participation in activation. As Ca^{2+}-binding to the low affinity sites is accompanied by little change in CD the triggering conformational change must be rather subtle, occurring essentially without a change in the overall secondary structure.

The Herzberg–Moult–James (HMJ) model

A model of the conformational change that occurs in TnC when Ca^{2+} binds to the low affinity sites has been proposed by Herzberg and colleagues (1986). They pointed out that the relative disposition of the helical segments in the Ca^{2+}-free N-terminal domain of the crystalline TnC is more compact than in the Ca^{2+}-filled C-terminal domain of TnC and in other Ca^{2+}-binding proteins. With the use of molecular modelling and on the assumption that the structure of the Ca^{2+}-bound form of the N-terminal domain should match that of the C-terminal one they deduced the putative structure of the completely Ca^{2+}-saturated TnC molecule. It appeared that when Ca^{2+} binds to sites I and II the major conformational transition is a movement of the B/C pair of helices away from the A/D pair thereby exposing a patch of hydrophobic residues possibly providing a binding site for TnI in the presence of Ca^{2+} and not in its absence. Herzberg and colleagues (1986) have further calculated that such a conformational transition would not encounter a large energy barrier and thus it would be consistent with the known fast kinetics of Ca^{2+}-binding and dissociation at these sites.

Further insights into the mechanism of the Ca^{2+}-induced conformational changes within a single helix-loop-helix Ca^{2+}-binding unit were provided by Richardson and Richardson (1988). They pointed out that the two helices in the Ca^{2+}-binding unit are offset by about 9 Å and form a tight contact at the crossing point which they called a lap-joint. They further suggested that the contact point may work as a pivot that couples a change in helix contact angle to an alteration of the loop conformation, and could therefore couple ion binding at the loop to overall tertiary structure change and *vice versa*.

The HMJ model and its more general version, the helix lap-joint of Richardson and Richardson, have many attractive features. They explain how a small local adjustment in the folding of the polypeptide chain associated with the coordination of the calcium ion of only 0.2 nm in diameter can be propagated to alter the structure 2 nm away, and how a rather large conformational change can be achieved without altering the secondary structure. The HMJ model explains the known Ca^{2+}-induced exposure of hydrophobic sites in

TnC resulting in an enhanced interaction with hydrophobic compounds such as phenothiazine, ANS (1-anilinonaphthalene-8-sulfonic acid) or phenylsepharose. It also provides a mechanism for the coupling between Ca^{2+}-binding and the interaction with TnI. Last but not least, as these models clearly specify changes in the side chain interactions they can be tested experimentally.

Experimental evidence for the HMJ model

The HMJ model has a strong support along several lines of evidence. The Ca^{2+}-induced relative movement of helical segments accompanied by exposure of some hydrophobic side chains has been postulated on the basis of the ^{1}H-NMR data (Levine *et al.*, 1977, 1978; Evans *et al.*, 1980). More recently Ingraham and Hodges (1988) have shown that in cardiac TnC Cys-84 located in helix D becomes approximately 2.4 times more reactive with iodoacetamide in the presence of Ca^{2+}. This result is qualitatively consistent with the Ca^{2+}-induced increase in the accessible surface area from 0.1 Å^2 to 50.9 Å^2 predicted by the HMJ model for the side chain of Gln-85 in turkey TnC, the residue homologous to Cys-84 in cardiac TnC. Similarly, Fuchs and colleagues (1989) have shown that in the presence of Ca^{2+} the kinetics of the reaction with DTNB of Cys residues in cTnC is 1.8 times faster and that the SH specific probe 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin labels predominantly Cys-84. Although the change in the reactivity was consistent with the model the overall rate of reaction was slow suggesting that even in the presence of Ca^{2+} Cys-84 is not fully exposed. This may indicate that the presence of the inactive site I in cTnC inhibits the full opening of the structure on Ca^{2+}-binding to site II.

Site-directed mutagenesis made further testing of the HMJ model possible. In a mutant rabbit skeletal TnC in which Cys was substituted for Ala-57 in the C-helix and the indigenous Cys-98 was replaced by Leu Ca^{2+} decreased the lifetime of the fluorescence probe 1,5-IAEDANS [N-iodoacetyl-N′-(5-sulfo-1-naphthyl) ethylenediamine] attached at Cys57 indicating an increased exposure of the probe to solvent (Wang *et al.*, 1990a). Similar results were obtained for fluorescently-labelled TnC45, a mutant containing a sole Cys at position 45 in the B-helix (Wang *et al.*, 1990b). Both studies are consistent with the Ca^{2+}-induced movement of the B- and C-helices proposed in the HMJ model. More direct evidence of the Ca^{2+}-induced change in the interhelical distance came from the studies on a mutant TnC (TnC12/49) with Cys residues at positions 12 and 49 in the A-helix, and in the segment linking helices B and C, respectively. Ca^{2+}-binding to the low affinity sites caused a decrease in the excimer emission of pyrene-maleimide labelled TnC12/49 and in the resonance energy transfer efficiency of TnC12/49 labelled with 1,5-IAEDANS and DDP-Mal [N-(-dimethylamino-3,5-dinitrophenyl)-maleimide] (Wang *et al.*, 1991). The resonance energy transfer data indicated an increase in the separation of the Cys's by about 13 Å in excellent agreement with the 11 Å increase predicted by the HMJ model.

Physiological significance

The question remains whether the conformation change occurring in consonance with the HMJ model is physiologically relevant. Two papers appearing at the same time addressed this question. Fujimori and colleagues (1990) produced two mutants of chicken skeletal TnC in which either Gly-57 in the C-helix or Glu-88 in the D-helix were replaced with Lys, thus introducing a salt bridge in place of the repulsive charge–charge interaction between the two glutamates. These mutants had decreased affinity for Ca^{2+} at the low affinity sites; when exchanged for natural TnC in a skinned muscle fibre, they could still activate tension generation, but at slightly higher [Ca^{2+}]. These results are consistent with the inhibition by the salt bridge of the putative Ca^{2+}-induced separation between the C and D helices. Perhaps more striking were the results obtained with a mutant of rabbit skeletal TnC that contained cysteine residues at position 48 in the loop connecting helices B and C, and position 82 in the D-helix (designated as TnC48/82) (Grabarek *et al.*, 1990). The location of the two genetically-engineered cysteines was such that a disulphide bond could be expected to form between them thus preventing effectively the separation of the B/C linker from helix D and the opening of the hydrophobic cleft in the N-terminal domain. The two cysteines were indeed spontaneously oxidized to form a disulphide which caused a decrease in affinity for Ca^{2+} and for TnI. Most important, the oxidized TnC48/82 binds to reconstituted thin filaments but its ability to activate myofibrillar ATPase activity is decreased by 80%, whereas that of the reduced TnC48/82 is indistinguishable from native TnC. Further studies have shown that the disulphide has little effect on the α-helix content but makes the N-terminal domain inaccessible for TnI (Gusev *et al.*, 1991). These results are readily interpretable in terms of the HMJ model. By preventing the movement of the B/C linker and presumably the B–C helices the disulphide stabilizes the Ca^{2+}-free form of the molecule relative to the Ca^{2+}-saturated form, resulting in the observed decrease in Ca^{2+}-affinity. Whether or not Ca^{2+} is bound at the low affinity sites, residues in the interhelical hydrophobic cleft remain inaccessible for interaction with TnI, rendering the oxidized TnC48/82 incapable of activating myofibrillar ATPase activity.

C-terminal domain

The role of the C-terminal domain in regulation and its interaction with TnI was examined with the use of a mutant (TnC98/122) containing a Cys at residue 122 in addition to the indigenous Cys98 (Grabarek *et al.*, 1991). It was found that a disulphide bond between the two Cys's is readily formed resulting in much reduced affinity between the oxidized TnC98/122 and TnI.

At low concentrations TnC98/122 does not confer Ca^{2+}-sensitivity on TnC-deficient myofibrils owing to its failure to associate with myofibrillar thin filaments. At higher concentrations partial regulatory activity was obtained, indicating that the regulatory capacity of oxidized TnC98/122 is in part retained. These results and those discussed above show that although both domains of TnC interact with TnI, the C-terminal domain appears to serve primarily as a binding domain and provides most of the free energy of binding, whereas the N-terminal domain serves the regulatory function in spite of a much weaker interaction with TnI. If the inhibitory segment of TnI (residues 96–116) indeed interacts with both domains of TnC, then the interaction with the C-terminal domain must have a different effect on its conformation than the interaction with the N-terminal domain, only the latter inducing the conformation of TnI in which it is not capable of interacting with actin and inhibiting actomyosin ATPase.

Conclusions

There is now a large body of evidence in support of the view that Ca^{2+} binding to the low affinity sites of TnC induces a movement of helices B and C away from helices A and D, thus opening a hydrophobic cavity, the site of interaction with TnI. Another site of similar structure is formed by the helical segments in the C-terminal domain. Both sites appear to interact with the inhibitory segment of TnI. Whereas the interactions at both sites are necessary for the full regulatory activity of TnC, the interaction at the C-terminal domain stabilizes the complex and that involving the N-terminal domain is directly linked to the release of inhibition. In the absence of Ca^{2+} the inhibitory region of TnI would preferentially bind to actin and on Ca^{2+} binding to sites I and II it would switch to the site in the N-terminal domain of TnC. Detachment of TnI from actin gives rise to further events in thin filament regulation.

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