Calcium-Induced Conformational Changes in the Troponin–Tropomyosin Complexes of Skeletal and Cardiac Muscle and Their Roles in the Regulation of Contraction–Relaxation

WILLIAM D. MCCUBBIN and CYRIL M. KAY*

Medical Research Council Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Received November 26, 1979

Some of the most interesting and challenging questions in biochemistry today relate to the way in which calcium triggers the molecular events that lead to muscle contraction. Let us consider, as background to these questions, some basic aspects of the structure of muscle.

Figure 1, modified after Lehninger,[1] depicts a schematic diagram of a striated muscle, the main features of which could apply equally well to either cardiac or skeletal muscle. Like other tissues, vertebrate muscle consists of cells, but muscle cells are very elongated and are often referred to as muscle fibers. At each end of the muscle, the fibers are usually attached, via a tendon, to the skeleton on which the muscle acts. The muscle fiber is, in turn, composed of many long thin myofibrils, each of which shows a regular repeating pattern of cross striations. These repeating units are known as sarcomeres and are separated from each other by Z disks. Myofibrils are composed of smaller myofilaments, which are of two kinds: the so-called thick filaments, about 150 Å in diameter and 1.5–1.6 μm long, are made up of an array of myosin molecules, while the thin filaments, about 60 Å in diameter, contain polymerized actin molecules as the major protein.

There is an overlapping of the thick and thin filaments only in the central region or A band. In this overlap region, electron micrographs show the presence of interfilamentous cross-bridges extending from the surface of the thick filaments toward the thin filaments. Muscle contraction is due to a relative motion of one type of filament along the other, with no appreciable change in the lengths of the filaments themselves. This relative sliding motion is the result of the constant formation and disruption of the cross-bridges between the two myofilament classes. In molecular terms, the cross-bridges which are composed of heads of the myosin molecule project outward to contact the actin molecules in the thin filament; concerted movement of the attached heads pushes the opposed sets of thin filaments toward each other, and the fibril shortens.

The signal that initiates these events is the liberation of calcium ions from membrane-bound storage sites by the arrival of a nerve impulse at the muscle. The signal is detected and acted on by two further sets of protein molecules, tropomyosin and troponin, which are positioned along the actin strand and with it constitute the thin filament.[2-4]

The precise disposition of these proteins in the thin filament is depicted in Figure 2, modified after Ebashi et al.,[5] in which the principal protein, fibrous or F-actin, is represented as two strands of pearls (each pearl being a molecule of globular or G-actin) wound around each other. Tropomyosin molecules are depicted as rods which lie end-to-end in the grooves of the actin strands, each spanning the length of 7 actin monomers.[6] Positioned at intervals of about 400 Å along the thin filament are the units of the troponin complex, each unit consisting of one Ca(II)-binding subunit (TN-C), one inhibitory member (TN-I) and one tropomyosin binding subunit (TN-T). It has been established that TN-I inhibits the Mg(II) activated ATPase (adenosine triphosphatase) of actomyosin, thus preventing contraction; TN-C is a Ca(II)-binding protein which is responsible for reversing the TN-I induced inhibitory effect; and TN-T localizes the troponin complex on tropomyosin.

We are now in a position to discuss a model for contraction–relaxation. In resting muscle, the free Ca(II) concentration is lower than 10^{-7} M and the concerted action of the tropomyosin–troponin system results in inhibition of the Mg(II)-stimulated ATPase activity of the myofibril. Depolarization of the muscle membrane by the nerve impulse initiates contraction by releasing stored Ca(II) from the membranous compartments of the sarcoplasmic reticulum (SR) and raising its concentration in the sarcoplasm to about 10^{-5} M. At this concentration, Ca(II) is bound to the TN-C component of the troponin complex, the inhibition of the fibrillar ATPase is released, and contraction accompanies the high rate of ATP hydrolysis. Once stimulation is over, Ca(II) is sequestered again within the sarcoplasmic reticulum, restoring the free Ca(II) concentration in the sarcoplasm to the resting level, and Ca(II) leaves the troponin complex.[7] This reinvokes the inhibitory action of the regulatory protein system and the muscle

Cyril M. Kay was born in Calgary, Alberta, and carried out his undergraduate studies in biochemistry at McGill University and his doctoral work at Harvard. After a postdoctoral stint in the biochemistry department at Cambridge University and a year at Eli Lilly & Co., Indianapolis, he joined the University of Alberta in 1958. He is presently Professor of Biochemistry and co-director of the MRC Group in Protein Structure and Function at the University of Alberta.

William D. McCubbin was born in Scotland and received both his B.Sc. and Ph.D. degrees in Chemistry from the University of Glasgow. He joined the laboratory of C. M. Kay in 1965 and is presently a research associate in the MRC Group in Protein Structure and Function.

(1) A. L. Lehninger, "Biochemistry", Worth Publishers Inc., New York, 1970.
(2) S. Ebashi and M. Endo, *Prog. Biophys. Mol. Biol.*, 18, 123 (1968).
(3) A. Weber and J. W. Murray, *Physiol. Rev.*, 53 (1973).
(4) S. Ebashi, *Essays Biochem.*, 10, 1–36 (1974).
(5) S. Ebashi, M. Endo, and I. Ohtsuki, *Q. Rev. Biophys.*, 2, 351 (1969).
(6) J. Hanson and J. Lowey, *J. Mol. Biol.*, 6, 48 (1963).
(7) W. Hasselbach, *Prog. Biophys. Biophys. Chem.*, 14, 167 (1964).

0001-4842/80/0113-0185$01.00/0

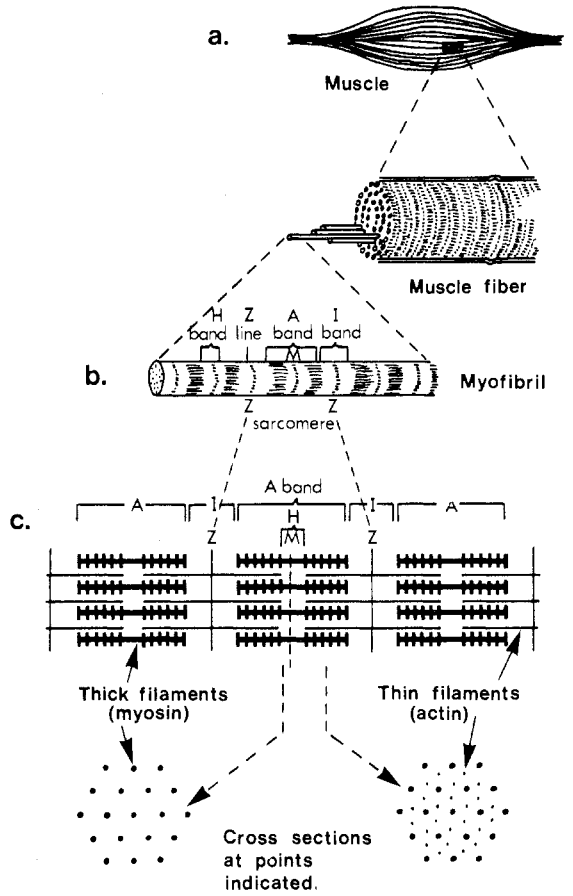

Figure 1. Gross structure of striated muscle. (a) Whole muscle; (b) part of one myofibril showing cross striations; (c) substructure showing arrangement of myofilaments within one sarcomere. (Modified from Lehninger.[1])

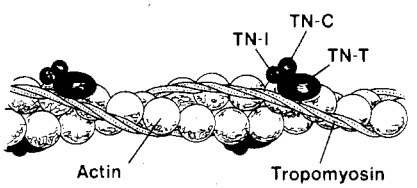

Figure 2. Model for the fine structure of the thin filament of muscle. (Modified from Ebashi et al.[5])

relaxes. Thus, by effectively controlling the enzymic activity of the myofibril, in response to changes in the free Ca(II) concentration, the regulatory protein system regulates the contraction–relaxation cycle of muscle.

A possible physical model for this system, largely worked out by Huxley and Parry,[8,9] suggests that in the relaxed state the binding site on the F-actin monomers for the heads of the myosin molecule is physically excluded by the tropomyosin (TM) molecule. This state of affairs is presented diagrammatically in Figure 3, modified from Potter and Gergely,[10] which shows an end-on view of the thin filament. According to this steric hypothesis, in the relaxed state the TN-T subunit binds to tropomyosin and the inhibitory subunit binds to actin. Under these conditions, TM is in the blocking position and prevents the attachment of the myosin head to actin. Upon activation, i.e., release of Ca(II)

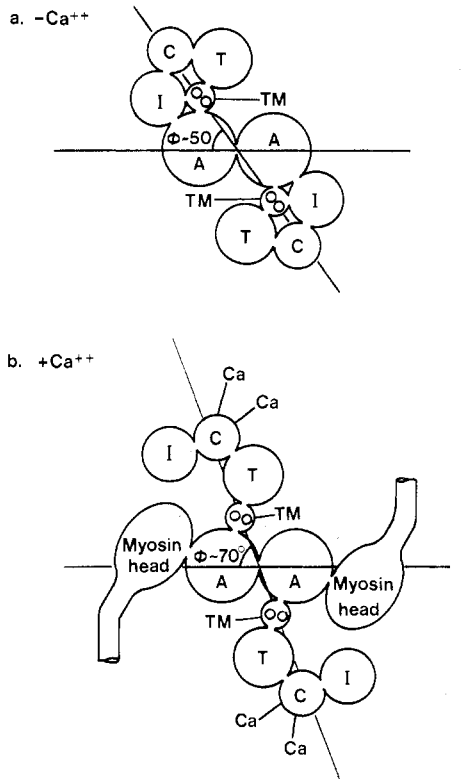

Figure 3. Model for the regulation of muscle contraction. The symbolism for the proteins is the following: actin, A; tropomyosin, TM; troponin I, I; troponin C, C; troponin T, T; the heavy meromyosin subfragment one portion of myosin, HMM S-1. Open areas between components indicate intermolecular interactions. (a) Positions of the proteins in relaxed muscle (pCa $\simeq$ 8). (b) Positions of the proteins in contracting muscle (pCa $\simeq$ 5). (Modified from Potter and Gergely.[10])

by the SR and combination of Ca(II) with TN-C, the TN-I-actin link is broken. The TN-I by moving away from the actin, or to a different position on the actin, allows TM to move from the blocking position on the actin to one nearer the groove of the two-stranded actin helix. This represents the "on" position, whereby the myosin heads now bind to actin, ATP is split, and the muscle contracts. Relaxation occurs as Ca(II) is taken up by the SR and removed from TN-C. Under these conditions, and by a reversal of the above processes, TM again moves to block the interaction of myosin with actin and to inhibit actomyosin ATPase activity. This is the "off" position and the cycle is ready to repeat itself.

Such a model must be regarded as tentative, since the structural states it invokes in the troponin subunits have yet to be directly demonstrated in clear visual images by electron microscopy or X-ray diffraction. As well, it was formulated on the assumption that the myosin attachment site and the tropomyosin position are on the same side of the actin groove, which may or may not be the case.

The trigger for the series of structural events that take place during regulation is the binding of Ca(II) by the TN-C member of the troponin complex, and, as will be noted below, this metal binding is accompanied by a conformational change in the protein molecule which is transmitted through the quaternary structure of the entire multiprotein relaxing system. To understand these processes better, we must take a closer look at some of the molecular properties of the TN-C system.

(8) H. E. Huxley, *Cold Spring Harbor Symp. Quant. Biol.*, **37**, 361 (1972).
(9) D. A. D. Parry and M. M. Squire, *J. Mol. Biol.*, **75**, 33 (1973).
(10) J. D. Potter and J. Gergely, *Biochemistry*, **13**, 2697 (1974).

The molecular weight of the polypeptide chain of TN-C from skeletal muscle, as established from amino acid sequence studies, is 17 840.[11] This is an acidic protein with an isoelectric point of 4.1–4.3. There are no tryptophan residues in the molecule, but two tyrosine moieties are present. The protein has a high phenylalanine:tyrosine ratio, 5:1, which shows up in the absorption spectrum, with five clearly defined maxima due to phenylalanine being obvious. It was demonstrated by the technique of equilibrium dialysis that TN-C has two classes of Ca(II)-binding sites: two sites which have a high affinity for Ca(II) but also bind Mg(II) competitively (Ca(II)–Mg(II) sites, $K_{Ca} = 2.1 \times 10^7\ M^{-1}$, $K_{Mg} = 5 \times 10^3\ M^{-1}$) and two sites which bind Ca(II) specifically but with a lower affinity (Ca(II)-specific sites, $K_{Ca} \simeq 3.2 \times 10^5\ M^{-1}$).[12] It was also found that there is an approximately one order of magnitude increase in the affinity of TN-C for all four Ca(II) upon interaction with TN-I, or when calcium binding was measured in native troponin (i.e., troponin which had not been fractionated into its subunits).

The Conformational Change Induced in TN-C

The far-UV circular dichroism (CD) spectrum of TN-C revealed that the conformation of the protein depends to a large extent on the Ca(II) concentration. The spectra were typical of an α-helical-containing protein with minima near 221 and 208 nm. With addition of Ca(II), there was an approximately 60% increase in the value of the ellipticity at 221 nm, $(\theta)_{221nm}$, suggesting a rather dramatic conformational change.[13] The induction of this large conformational change upon Ca(II) addition was corroborated by hydrodynamic measurements on this system, where it was found that Ca(II) caused an increase in the intrinsic sedimentation constant, $s_{20,w}$, from 1.84 to 2.5 S. Moreover, it was found that the weight-intrinsic viscosity, $[\eta]$, decreased from 8.5 to 3.3 mL/g on Ca(II) addition, while the molecular mass remained constant at 18 000 daltons. The increase in $s_{20,w}$ and parallel decrease in $[\eta]$ at constant M upon addition of Ca(II) implies a compacting of the molecule and an overall increase in ordered structure.

We also showed that other metals, notably Mg(II), Cd(II), and Ba(II), produced a conformational change in TN-C, representing about 50–75% of the change induced by Ca(II) under the same conditions. The binding constants for these metals were demonstrated to be of the order of $10^3\ M^{-1}$, several orders of magnitude less than for Ca(II).[14]

One can also use fluorescence measurements to follow the conformational change in TN-C. Since the protein contains no tryptophan, the fluorescence spectrum is essentially that of tyrosine. When the tyrosine residues were excited at 276 nm and the resulting emission was monitored at 306 nm, there was a pronounced increase in the fluorescence intensity of the tyrosine residues as Ca(II) was added, paralleling completely the CD observations. The increase in fluorescence intensity may be directly correlated with the considerable increase in helix content in the protein molecule upon adding Ca(II).[15]

We have also used UV difference absorption spectroscopy to demonstrate a Ca(II)-induced change in TN-C. The difference spectra are characterized by maxima near 287 and 277 nm due to perturbation in the environment of tyrosine. These positive peaks imply that the environment of one or more tyrosine residues has/have become more nonpolar, consistent with the compacting of the TN-C structure when binding Ca(II), accompanied by neutralization of negatively charged carboxyl groups. The maxima near 269, 265, 259, and 253 nm can be attributed to contributions from the phenylalanine vibrational fine structure. These peaks are more difficult to interpret since they represent the averaged environmental effects of the 10 phenylalanine residues in the TN-C molecule.[16]

What type of groups in the TN-C molecule might be implicated in Ca(II) binding? We demonstrated on the basis of the following observations that certain carboxylic acid functions were logical contenders. Some 40 out of 60 CO_2H groups in the TN-C molecule were converted into neutral amides by a two-step reaction—the Hoare–Koshland reaction—involving a water-soluble carbodiimide and an amine such as glycine amide in accordance with the reaction:

$$\mathrm{ProtCO_2^-} + \mathrm{H_2NCH_2C(O)NH_2} \xrightarrow[\text{pH } \sim 5]{\mathrm{R'N{=}C{=}N{-}R''}} \mathrm{ProtC(O)NHCH_2C(O)NH_2}$$

The number of CO_2H groups modified was determined by amino acid analysis, after acid hydrolysis to detect the increase in the amount of glycine. These derivatized TN-C samples were then studied by CD. It was found that the percentage change in $[\theta]_{221nm}$ was only some 8% upon adding Ca(II), as opposed to a 60% change on adding Ca(II) to native TN-C. Also, the baseline ellipticity value for the derivative in the absence of Ca(II) is some 3000° more negative than the value for native TN-C. It would thus appear that the introduction of 40 glycinamide residues, with the resulting removal of negative charge from the molecule, has partially induced a similar sort of conformational change as did Ca(II). Further, the derivatized molecule has lost most of its responsiveness to Ca(II) in terms of undergoing a much smaller conformational change than the native molecule. It should also be stated that the carboxyl-modified derivative, unlike native TN-C, was incapable of neutralizing the inhibitory effect of TN-I on the Mg(II)-activated ATPase of actomyosin.[14]

These results suggest that the Ca(II)-binding sites on TN-C include certain key carboxylate groups of aspartate and/or glutamate residues. Information about the environment of these CO_2H groups was obtained by carrying out conformational studies on this system as a function of pH. It was found that H_3O^+ ions will induce a similar conformational change in TN-C as does Ca(II) in terms of both the fluorescence change (a 30% enhancement) as well as the change in CD ellipticity (a 50% increase in negativity).[17] In both cases, from the midpoint of the titration curves, one can deduce

(11) J. H. Collins, J. D. Potter, M. J. Horn, G. Wilshire, and N. Jackman, *FEBS Lett.*, **36**, 268 (1973).
(12) J. D. Potter and J. Gergely, *J. Biol. Chem.*, **250**, 4628 (1975).
(13) A. C. Murray and C. M. Kay, *Biochemistry*, 11, 2622 (1972).
(14) W. D. McCubbin and C. M. Kay, *Biochemistry*, **12**, 4228 (1973).
(15) Y. Kawasaki and J. P. van Erd, *Biochem. Biophys. Res. Commun.*, **49**, 898 (1972).
(16) M. T. Hincke, W. D. McCubbin, and C. M. Kay, *Can. J. Biochem.*, **56**, 384 (1978).
(17) S. S. Lehrer and P. C. Leavis, *Biochem. Biophys. Res. Commun.*, **58**, 159 (1974).

that protonation of CO_2H groups with pK'_a values of 6.0 are producing the substantial increases in both parameters. This high pK' is not unusual for proteins, since several proteins with carboxyl groups having abnormally high pK values (>6) have been reported.[18] Such high pK values are associated with carboxylates located in regions containing adjacent carboxylates, particularly if the environment is somewhat hydrophobic. This is, in fact, the situation in TN-C which has a negative charge of about 30 at neutral pH with many such groupings of acid residues, as was noted when the amino acid sequence data became available.[8] It seems probable that these same carboxylates are involved in Ca(II) binding.

In the case of the tyrosine fluorescence, un-ionized carboxyl groups can quench tyrosine fluorescence if they are bonded to, or located near, tyrosine side chains.[19] The fluorescence enhancement of TN-C at low pH can be interpreted as being due to a decrease in the proximity of quenching groups caused by proton binding to carboxylates of $pK' = 6.0$. It is not possible to decide from this data if the groups with $pK' = 6$ are themselves the quenchers, or whether they regulate the proximity of other quencher groups to tyrosyl, because of the large conformational change which occurs in the same pH range.

In a CD and UV difference spectral study of the separate but similar effects of Ca(II) and H_3O^+ addition to TN-C, we confirmed the presence of this important class of carboxyl groups in TN-C that regulate Ca(II) binding and its expression as a conformational change.[16]

Propagation of the Conformational Change Induced in TN-C through the Relaxing System

So far we have described an interesting and large conformational change induced in the TN-C molecule by Ca(II) addition. If this conformational change is to have any physiological relevance, it must be demonstrated that although it originates in the TN-C moiety it is transmitted through the entire relaxing system. Potter[20] showed, in a sodium dodecyl sulfate poly(acrylamide) gel electrophoresis study of myofibrils, that the troponin subunits occur in a 1:1:1 ratio of TN-C to TN-I to TN-T, so that it is possible to prepare reconstituted troponin (TN-ICT) by adding these three components in an equimolar ratio. Furthermore, sedimentation velocity measurements can be used to substantiate complex formation. With TN-ICT, we showed that the observed $s_{20,w}$ of 3.05 S was larger than that of any individual component alone and further that its schlieren pattern, as observed in the ultracentrifuge, was characterized by a single symmetrical peak with no evidence of any slower moving peaks.[21]

As well, ATPase measurements with various members of the troponin complex present in the assay system were used as indicators of complex formation. As shown in Table I, TN-I inhibits the Mg(II)-activated ATPase of a synthetic actomyosin assay system independently of the Ca(II) concentration (forming a link between TM and actin, locking TM in the blocking position). Since

Table I
ATPase Measurements of Synthetic Actomyosin[a]

	ATPase activity, μmol of PO_4 s^{-1} g of actomyosin^{-1}	
	EGTA	Ca(II)
no troponin	8.3	8.1
troponin I	5.3	5.4
troponin I + C	8.1	8.2
troponin C + T	8.0	8.1
troponin I + C + T	2.0	8.4

[a] These measurements were made as described in ref 21. All assay systems contained: myosin, 0.60 mg; actin, 0.15 mg; tropomyosin, 0.04 mg; and troponin (TN-I, 0.06 mg; TN-IC, 0.11 mg; TN-CT, 0.14 mg; TN-ICT, 0.48 mg) in 10 mL of reaction mixture.

TN-I does not bind Ca(II), the inhibition would be Ca(II) insensitive. Addition of TN-C reversed the TN-I inhibition, even in the absence of Ca(II). This implies that in the presence of TN-C, the binding of TN-I to actin-TM must be altered relative to TN-I alone, since the combination of myosin with actin—presumably with the accompanying displacement of TM—can take place even in the absence of Ca(II). TN-ICT mimics natural troponin by conferring Ca(II) sensitivity on the Mg(II)-activated ATPase of synthetic actomyosin.[21]

Several complexes have been prepared between TN-C and the other two subunits, TN-I and TN-T, and the resulting materials were examined by CD as a function of Ca(II) concentration. It was clear in all cases that the secondary structures of these complexes were sensitive to Ca(II) and a conformational change, analogous to that induced initially in TN-C, could still be invoked by this cation. Furthermore, the binding constants obtained for Ca(II) were higher than those obtained for TN-C alone under similar conditions.[21,22] Ca(II) also produced a stabilizing effect on the various complexes produced in terms of resistance to thermal denaturation.

These CD measurements have also been extended to include tropomyosin. As mentioned earlier, the troponin complex is bonded to TM via the TN-T member. Demonstration of an interaction between TN-T and TM was initially shown by CD, and the secondary structure of these proteins was found to be insensitive to Ca(II). Ca(II) did, however, affect the structure of a system containing both TM and TN-C; in particular, it became apparent that the Ca(II)-induced change in TN-C, already demonstrated to occur in the ternary troponin complexes, was effective in a fully reconstituted relaxing system containing all the troponin subunits and TM.[23] All these facts, taken together with the persistence of the conformational change in TN-C through the entire troponin-TM complex, are consistent with the molecular model presented in Figure 3. Nonetheless, it should be noted that this model ignores possible conformational changes in the F-actin structure induced either through the tropomyosin–troponin system or through its interaction with the myosin heads. These possibilities may be particularly relevant if it should be proven that the postulated myosin attachment site and the tropomyosin position are not on the

(18) Y. Nozaki, L. C. Bunville, and C. Tanford, *J. Am. Chem. Soc.*, **81**, 5523 (1959).
(19) Review of G. Weber and F. W. J. Teale, "The Proteins", 1975, Academic Press, New York, Chapter 17, p 3.
(20) J. D. Potter, *Arch. Biochem. Biophys.*, **162**, 436 (1974).
(21) R. S. Mani, W. D. McCubbin, and C. M. Kay, *Biochemistry*, **13**, 5003 (1974).
(22) W. D. McCubbin, R. S. Mani, and C. M. Kay, *Biochemistry*, **13**, 2689 (1974).
(23) R. S. Mani, W. D. McCubbin, and C. M. Kay, *FEBS Lett.*, **52**, 127 (1975).

same side of the actin groove.

Besides sedimentation velocity and ATPase measurements, other approaches have been used to demonstrate specific interactions between various members of the relaxing system. For example, Head and Perry, using poly(acrylamide)–urea gels, demonstrated that TN-C and TN-I formed a strong complex, even in the presence of 6 M urea, if Ca(II) was present in the system, but not in its absence.[24]

Far- and near-UV CD measurements on a 1:1 mixture of cardiac TN-I and TN-T were effected in our laboratory, and the observed spectra were compared with theoretical ones generated for the two components, assuming they were simply present in an equimolar ratio but did not interact. Differences between the two spectra greater than 500° were taken as evidence for interaction, in which formation of a complex has caused secondary structure alterations in either or both of the proteins. In this specific case, an increased experimental negative ellipticity (relative to the theoretical) of 1000° at 220 nm provided definite evidence for a TN-T–TN-I interaction. As well, the near-UV CD spectrum showed evidence for an environmental alteration of some tyrosine(s) in the complex relative to the uncomplexed proteins.[25] This same interaction had earlier been demonstrated in a cross-linking study of skeletal troponin which revealed that TN-T–TN-I is a major product of the reaction utilizing dimethyl imidoesters.[26]

It is noteworthy that no such interaction could be demonstrated by CD if the two sulfhydryls of the cardiac TN-I component were not maintained in the free reduced state. A similar phenomenon has been observed by Horwitz et al. for the skeletal TN-I–TN-T interaction.[27] In these cases, it is conceivable that, unless SH groups are protected, nonphysiological disulfide formation (both intra- and intermolecular) of the various members of the troponin complex may occur when attempts are made to reconstitute such a complex from individual subunits, or even when dealing with native troponin. Such misassembled complexes may possess a degree of rigidity not permitting normal responsiveness to Ca(II).

Co-sedimentation studies are another effective way of demonstrating complexation and such an approach has been used to reveal interactions between TM and TN-I as well as between TM and TN-T. In this case, TM was mixed with either TN-I or TN-T in 0.5 M KCl; the mixture was then dialyzed to 0.1 M KCl (an ionic strength at which TM is soluble), which resulted in the precipitation of part of the protein. Gel electrophoresis of the precipitates indicated that, in addition to either TN-T or TN-I, TM was also present, thus demonstrating interprotein interaction.[28]

Chemical Nature of the Interaction Sites between Troponin Subunits

This brings us to the question of what is known about the sites of interaction of the various components with each other and with TM and actin. This is a hotly pursued research area because of three important developments: first, the amino acid sequences of all the skeletal TN components and of actin and TM are known; second, it is possible to produce, by controlled chemical and/or enzymatic digestion of the various protein components, chemically well-defined fragments of these molecules; and third, using affinity chromatography whereby one of the protein components is covalently linked to Sepharose, one can discern which of the various fragments of a given member of the troponin complex binds to the Sepharose-bound protein.

MCBP	(18-29)	AB:	cys	lys	ala	ala	asp	ser	phe	asp			his	lys	ala	phe
	(50-63)	CD:	ile	ASP	gln	ASP	lys	ser	GLY	phe	ILE	glu	glu	asp	GLU	leu
	(89-101)	EF:	gly	ASP	ser	ASP	gly	asp	GLY	lys	ILE	gly	val	asp	GLU	phe
TN-C	(26-39)	I:	phe	(ASP)	ala	(ASP)	(gly)	gly	(GLY)	asp	(ILE)	ser	val	lys	(GLU)	leu
	(62-75)	II:	val	(ASP)	glu	(ASP)	(gly)	ser	(GLY)	thr	(ILE)	asp	phe	glu	(GLU)	phe
	(101-114)	III:	phe	[ASP]	arg	asn	ala	[asp]	[GLY]	tyr	[ILE]	[asp]	ala	glu	[GLU]	leu
	(137-150)	IV:	gly	[ASP]	lys	ASP	asn	[asp]	[GLY]	arg	[ILE]	[asp]	phe	asp	[GLU]	phe

Figure 4. Sequences of rabbit TN-C and carp component 3 MCBP (parvalbumin) in the segments corresponding to the Ca(II)-binding sites of MCBP. Residues (in CD and EF) that are involved in Ca(II) binding are underlined. Residues identical in five or more regions are capitalized. The circles correspond to amino acid identities in sites I and II of TN-C while the squares refer to amino acid identities in sites III and IV. (Modified from Collins et al.[11])

It was demonstrated, using such an approach, that a cyanogen bromide fragment of TN-I (encompassing residues 124–145) bound to actin and at the same time inhibited actomyosin ATPase. This same fragment also bound to TN-C, which correlates with the fact that TN-C will neutralize the inhibition of the actomyosin ATPase by TN-I. A second binding site for TN-C on the TN-I molecule is at the N terminus since it was shown, also by affinity chromatography, that a cysteine-cleavage fragment (residues 24–74) bound to TN-C.[29] By a similar approach, the TN-I binding site on the TN-C molecule was implicated to be in a cyanogen bromide peptide of this molecule running from residues 83 to 134.[30]

Binding studies of a number of TN-T fragments to TN-C immobilized on a Sepharose column revealed that the portion of the TN-T structure responsible for its calcium-sensitive interaction with TN-C resides in the C-terminal section of the molecule from residues 159 to 259. This includes the highly basic C-terminal segment of sequence from residues 221 to 259 plus a region with mixed-charged properties extending from residues 159 to 220. As well, it was shown, in terms of the TN-T–TM interaction, that the portion of the TN-T molecule binding to TM encompasses residues 71–151, a highly helical section of the TN-T molecule.[31] The interaction probably involves a multi-α-helical complex between the two molecules, involving carboxyl side chains of glutamic and aspartic acids over a limited surface area of the double-stranded coiled coil of TM. Relevant to this point is the finding that TN-T appears to be an elongated molecule from hydrodynamic data (ultracentrifuge and fluorescence depolarization).[25,32]

(24) J. F. Head and S. V. Perry, *Biochem. J.*, **137**, 145 (1974).
(25) M. T. Hincke, W. D. McCubbin, and C. M. Kay, *Can. J. Biochem.*, **57**, 768 (1979).
(26) S. E. Hitchcock, *Biochemistry*, **14**, 5162 (1975).
(27) J. Horwitz, B. Bullard, and D. Mercola, *J. Biol. Chem.*, **254**, 350 (1979).
(28) R. Dabrowska, Z. Podlubnaya, E. Nowak, and W. Drabikowski, *J. Biochem.*, **80**, 89 (1976).
(29) H. Syska, J. M. Wilkinson, R. J. A. Grand, and S. V. Perry, *Biochem. J.*, **153**, 375 (1976).
(30) R. A. Weeks and S. V. Perry, *Biochem. J.*, **173**, 449 (1978).
(31) J. R. Pearlstone and L. B. Smillie, *Can. J. Biochem.*, **56**, 521 (1978).
(32) F. G. Prendergast and J. D. Potter, *Biophys. J.*, **25**, 250a (1979).

This asymmetric nature is similar to TM and may be of functional significance, as it would undoubtedly aid in binding to the TM molecule.

Location of the High- and Low-Affinity Ca(II)-Binding Sites in TN-C

When Collins established the primary structure of TN-C he aligned four segments of the TN-C structure which have similar sequences to the already known Ca(II)-binding sites of the protein parvalbumin, demonstrated by X-ray crystallography;[33] this is shown in Figure 4. These regions in TN-C are remarkably homologous to those of parvalbumin, both in terms of the amino acids involved in Ca(II) binding and in correspondence of the adjacent residues, suggesting that these four regions in TN-C are the probable Ca(II)-binding sites and, further, that these four regions probably have a similar tertiary structure to the so-called "EF and CD hands" of parvalbumin.[11] It was then suggested that each of these four regions of TN-C arose by gene duplication of the "EF hand". Presumably the "EF hand", or "helix–loop–helix" structure, is the primordial gene which duplicated several times to yield identical copies with a small amount of mutation with time.

Examination of the four postulated Ca(II)-binding sites in TN-C reveals that site I more closely resembles site II, while site III more closely resembles site IV (the numbering begins from the N terminus of the molecule). There are six identical amino acid residues between sites I and II, including two or more glycines and six identical residues between sites III and IV. Which are the high-affinity sites and which the low?

Recently, the nature of all four regions has been unambiguously resolved by studying the Ca(II)-binding properties of fragments of TN-C. Leavis et al. used trypsin, thrombin, and cyanogen bromide to cleave TN-C at different points along its polypeptide chain.[34] The resulting fragments contained one or more of the four binding regions (I to IV), and they were studied by CD and fluorescence and also in terms of their Ca(II) binding. The largest change in CD and fluorescence occurred with the fragments containing sites III and IV, of which the major contribution to the spectral changes arose from site III—these then are the high-affinity sites. Fragments containing only sites I and II showed no spectral change upon Ca(II) binding—these are the low-affinity sites. Fragments containing the two Ca(II)–Mg(II) sites (the high-affinity ones) had a strong affinity for Ca(II), 2×10^8 M^{-1}; they could also bind Mg(II). However, cleaving the polypeptide chain so that regions III and IV were on separate fragments changed the characteristics of the high-affinity sites. Now, they could only bind Ca(II) weakly ($\sim 10^5$ M^{-1}) and they failed to bind any Mg(II) at all. This emphasizes the importance of tertiary structure (relationships between different regions of the same molecule) for defining the overall properties of the binding sites.

Since there are both high- and low-affinity Ca(II) sites in TN-C, it was of interest to see if one could differentiate, on the basis of the optical techniques used, between the two classes of sites, i.e., could one detect respective contributions from each class of sites to the conformational change? To answer this question, we carried out a CD Ca(II) titration for skeletal TN-C at neutral pH by monitoring $[\theta]_{221nm}$ as a function of the addition of incremental amounts of calcium. A plot was made of the fraction of the total conformational change elicited by Ca(II) against the negative logarithm of the free Ca(II) concentration, pCa(II). Analysis of the data by a computer program and making the simplifying assumption that the binding sites in any one class are equivalent, independent, and non-interacting revealed two distinct classes of binding sites. It was found that about 70% of the conformational change was elicited by the high-affinity Ca(II)-binding sites ($K \simeq 10^7$ M^{-1}) and some 25% of the conformational change was produced by the low-affinity binding sites ($K \simeq 10^5$ M^{-1}).[16] Similar experiments were carried out by Johnson and Potter who monitored tyrosine fluorescence as well as the CD change and found biphasic responses in these parameters, suggestive of a major contribution from the high-affinity Ca(II)–Mg(II) sites, with a minor contribution from the low-affinity Ca(II)-specific sites.[35]

Physiological Implications

In order to obtain a better understanding of how Ca(II) binding to TN-C releases the inhibitory effect of the troponin complex and TM, we must know which Ca(II)-binding sites on TN-C are the regulatory ones. Work with myofibril preparations indicated that the Ca(II) concentration at which their ATPase activity is activated was independent of the concentration of Mg(II) and that it became maximal only when all four Ca(II)'s were bound.[12] Hence, only the Ca(II)-specific sites appear to be involved in regulation.

What does this mean in terms of the structural changes in TN-C that we are observing in vitro? Clearly, the concentrations of intracellular Ca(II) and Mg(II) (1–5 mM) are sufficiently high that the high-affinity Ca(II)–Mg(II) sites would be occupied by either Ca(II) or Mg(II) even during relaxation, i.e., the major conformational change we observe by CD or fluorescence always exists, even in the relaxed state. The physiologically important changes are probably represented by the smaller increases in CD and tyrosine fluorescence which occur when Ca(II) binds to the Ca(II)-specific regulatory sites.

This point of view has been substantiated by Potter's studies wherein he has used a conformational probe that is apparently specific for the Ca(II)-specific regulatory sites. Skeletal TN-C was labeled with dansylaziridine at methionine-25 near site I, and the bound dansylaziridine was used as a fluorescent reporter group for any conformational changes that might occur at site I upon adding Ca(II).[36] It was found that the fluorescence of the derivative was enhanced approximately 2-fold when Ca(II) bound to the Ca(II)-specific sites, and this was accompanied by a 10-nm blue shift. At the same time there was no alteration in the conformation of TN-C introduced by the label—it still bound 4 mol of Ca(II) and it underwent the biphasic Ca(II)-induced CD conformational change. The midpoint of the fluorescence-enhancement transition occurred at a

(33) R. H. Kretsinger and C. E. Nockolds, *J. Biol. Chem.*, **248**, 3313 (1973).
(34) P. C. Leavis, S. S. Rosenfeld, J. Gergely, Z. Graberck, and W. Drabikowski, *J. Biol. Chem.*, **253**, 5452 (1978).
(35) J. D. Johnson and J. D. Potter, *J. Biol. Chem.*, **253**, 3775 (1978).
(36) J. D. Johnson, J. H. Collins, and J. D. Potter, *J. Biol. Chem.*, **253**, 6451 (1978).

$K_{Ca(II)}$ value of 4×10^5 M^{-1}, suggesting that it was monitoring a low-affinity Ca(II)-binding site.

Potter has also used this dansylaziridine derivative to measure the rate at which the Ca(II) specific site induced conformational change occurs. He showed by stopped-flow fluorimetry that Ca(II) bound to both classes of sites and produced the structural and fluorescence change characteristic of this binding, within the 2.4-ms mixing time of the instrument. However, the rates of Ca(II) removal from these sites were very different. A very slow ($t_{1/2}$ ~700 ms) removal of calcium from the high-affinity sites was accompanied by a 10% increase in fluorescence, whereas a fast ($t_{1/2}$ 2–3 ms) removal of this cation from the Ca(II)-specific sites produced the large decreases in fluorescence characteristic of this process. Since the time span of events leading to contraction and relaxation in fast skeletal muscle is of the order of 50 ms, it is obvious that the conformational changes induced by Ca(II) removal from the high-affinity sites are too slow to be directly involved with the regulatory process. Since the Ca(II)-removal rate and the resulting conformational changes from the low-affinity sites are very rapid, it seems entirely plausible that these low-affinity sites are indeed the regulatory ones. The high-affinity sites which are probably always occupied by either Ca(II) or Mg(II) in vivo presumably stabilize the structure of the protein to maintain it in a conformation which is ready for the regulatory event of Ca(II) exchange with the Ca(II)-specific regulatory sites.[37]

Cardiac Regulatory System

Earlier studies performed in our laboratory concerning bovine cardiac troponin, its components, and their interactions[38-41] suggested that regulation of cardiac muscle contraction could be explained qualitatively by the same model proposed for the skeletal muscle system. There are, however, certain differences, perhaps the most notable being that the cardiac TN-C molecule binds only three Ca(II) ions in place of four: it lacks one of the low-affinity Ca(II)-binding sites, notably site I in the skeletal TN-C nomenclature, since it possesses two fewer aspartic acid residues at the level of this site.

Further evidence that the two systems are regulated in a similar fashion was demonstrated by preparing hybrid complexes of troponin using both skeletal and cardiac subunits and examining their CD and biological activity. It was found that for any one ternary complex the observed ellipticity at 220 nm was greater than the theoretical and, furthermore, addition of Ca(II) caused a further increase in negative ellipticity.[42] These results indicate that interprotein interactions occur among the hybrid components and, as well, Ca(II) addition causes a conformational change in the TN-C member which is transmitted through the entire troponin complex, in agreement with the observations obtained in either the exclusively cardiac or skeletal ternary complexes.

Bioassay studies were also revealing in showing that both skeletal and cardiac TN-I have inhibitory activity in the skeletal and cardiac actomyosin systems. It was noted that while each TN-I component was operative in both synthetic actomyosin systems, a given TN-I was most active with its "parent" actomyosin. It is also noteworthy that cardiac TN-I is a less effective inhibitor against cardiac synthetic actomyosin than is skeletal TN-I vs. its parent actomyosin system.[41] This finding of a smaller degree of inhibition of cardiac actomyosin ATPase by cardiac TN-I may have physiological relevance since cardiac muscle must function continuously, without an opportunity for rest and recovery.

Phosphorylation of Myosin from Striated and Cardiac Muscle

The discussion thus far has focused on vertebrate striated and cardiac muscle, where regulation is at the level of the thin filament proteins. In contrast, smooth-muscle regulation has been found to be associated with the presence of a specific light chain (or small subunit) on the head of the myosin molecule in the thick filament. It seems that the triggering of the actin–myosin interaction in smooth muscle is brought about by a calcium-dependent phosphorylation of the 20 000 dalton light chain of the myosin by a specific light-chain kinase.[43] Dephosphorylation occurs through the action of a phosphatase which is not calcium sensitive and is present in approximately equivalent amounts to the kinase. However, despite this finding of calcium-dependent phosphorylation as the trigger for actomyosin ATPase activity in smooth muscle and the demonstration of calcium-sensitive light-chain kinases in both striated and cardiac muscle[44,45] distinct from that in smooth muscle, no link between myosin phosphorylation and actin-activated ATPase activity in either skeletal or cardiac muscle has been observed to date.

Concluding Remarks

We have documented some of the salient observations linking the Ca(II)-induced conformational change in TN-C and the transmission of this transition through the entire relaxing system to the regulation of muscle contraction. Obviously the story is still far from complete, and many important questions remain. For example, how does the TM molecule move in the actin groove upon activation? Is it due solely to the release of a troponin restraint, or the result of a conformational change in the TM itself? Is the role of the F-actin polymer merely a structural passive one or are there subtle changes in conformation induced in the actin monomers by the Ca(II)–troponin interaction? What is the general shape of the troponin complex in vivo? Preliminary hydrodynamic measurements from our laboratory suggest that TN is not a globular molecule but rather an elongated or "knobby" one.[46] What are the requirements at the molecular level for high- and low-affinity Ca(II) binding as well as metal-ion specif-

(37) J. D. Johnson, S. C. Charlton, and J. D. Potter, *J. Biol. Chem.*, **253**, 3497 (1979).
(38) L. D. Burtnick, W. D. McCubbin, and C. M. Kay, *Can. J. Biochem.*, **53**, 15 (1975).
(39) L. D. Burtnick, W. D. McCubbin, and C. M. Kay, *Can. J. Biochem.*, **53**, 1207 (1975).
(40) L. D. Burtnick, W. D. McCubbin, and C. M. Kay, *Can. J. Biochem.*, **54**, 546 (1976).
(41) L. D. Burtnick and C. M. Kay, *FEBS Lett.*, **65**, 234 (1976).
(42) M. T. Hincke, W. D. McCubbin, and C. M. Kay, *FEBS Lett.*, **83**, 131 (1977).
(43) J. V. Small and A. Sobieszek, *Eur. J. Biochem.*, **76**, 521 (1977).
(44) W. T. Perrie, L. B. Smillie, and S. V. Perry, *Biochem. J.*, **135**, 151 (1973).
(45) N. Frearson, B. W. W. Focant, and S. V. Perry, *FEBS Lett.*, **63**, 27 (1976).
(46) D. M. Byers, W. D. McCubbin, and C. M. Kay, *FEBS Lett.*, **104**, 106 (1979).

icity? An interesting and unique approach to this problem is being made through the chemical synthesis of Ca(II)-binding site III and analogues thereof and a comparison of the binding characteristics of the synthetic fragments.[47]

Methods for the crystallization of the individual troponin subunits and the native complex will need to be developed or improved upon, since X-ray analysis holds promise of providing the ultimate picture of the multiprotein complex and the allosteric-type interactions its members undergo when TN-C binds Ca(II). Spectroscopic techniques such as CD, fluorescence, and NMR will, no doubt, be applied in more sophisticated ways, perhaps concentrating on the intact protein complex rather than on isolated subunits.

An exciting development in this regard concerns recent NMR work in which our colleagues have observed dramatic shifts induced in the ^{1}H NMR spectra of both parvalbumin and a fragment of TN-C containing the high-affinity binding site III, when the paramagnetic lanthanide ions praseodymium (Pr(III)) and ytterbium (Yb(III)) are used as analogues of Ca(II).[48] From an analysis of the shifts and the relaxation times for the shifted resonances, they are extracting the three-dimensional structure of the amino acids surrounding the metal-ion binding sites in solution.

We can also expect to see great advances in the elucidation of the role of some of the fine-tuning aspects of the Ca(II) regulation, e.g., the exploration of the hypothesis that the protein parvalbumin (now found in skeletal muscle of higher animals) acts in concert with TN-C as a soluble relaxing factor. All in all, we are on the threshold of some exciting developments in the structure–function relationships of this fascinating multiprotein complex.

We thank our co-workers cited herein for their invaluable contributions. We also express our appreciation to the Medical Research Council of Canada for their support of the work from our laboratory presented in this Account.

(47) R. E. Reid and R. S. Hodges, *J. Biol. Chem.*, in press.

(48) L. Lee, B. D. Sykes, and E. R. Birnbaum, *FEBS Lett.*, **98**, 169 (1979).