THIN FILAMENT PROTEINS AND THIN FILAMENT-LINKED REGULATION OF VERTEBRATE MUSCLE CONTRACTION

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I. INTRODUCTION

This review deals with the regulatory system of striated muscle. In current parlance, the term regulatory system refers to those proteins that are located in the so-called thin filaments of muscle. The main constituent of these thin filaments is actin, and according to the sliding filament model of muscle contraction the contraction of muscle is due to the interaction of myosin located in the so-called thick filaments with actin. The interaction of actin and myosin is considered to be a cyclic process accompanied by the hydrolysis of ATP in which a certain portion of the myosin molecule, the so-called head, attached to actin undergoes a change in its relation to the thin filament, sometimes referred to as attitude, detaches and is again ready to accept another ATP molecule to start a fresh cycle. This conceptual framework rests on the discoveries of the ATPase activity of myosin pioneered by Engelhardt and Ljubimova,[1] the separate existence of actin and myosin as distinct entities,[2] the localization of myosin and actin in discrete filaments,[3-5] and the recently elaborated scheme of ATP hydrolysis by myosin (compare References 6 to 8).

For some time after the essentials of the dual sliding filament model had been established, it remained something of a puzzle how the electric depolarization of the plasma membrane of the muscle cell elicited by a nerve impulse led to activation of the contractile apparatus. The early work of Heilbrunn and Wiercinsky,[9] showing that microinjection of Ca^{2+} into a muscle cell led to contraction, suggested the participation of calcium ions in the activation process. This view, however, remained dormant for almost a decade until it became clear that the concentration of myoplasmic Ca^{2+}, which in turn is regulated by the sarcoplasmic reticulum, controls in some way the interaction of actin and myosin, the activity of the actomyosin ATPase, and ultimately the development of force and contraction in muscle.

Our understanding of the problem of regulation of muscle contraction entered a new phase with the discovery by Ebashi and colleagues[10-14] of a protein system that was necessary for the Ca^{2+} sensitivity of actomyosin. It is at this point that the detailed examination of the problem in our review begins. We shall discuss the components of the regulatory protein system, viz., tropomyosin, and the troponin complex. The localization of tropomyosin in the thin filaments was suggested by Hanson and

Lowy.[15,16] Ebashi and colleagues[12,13] made the logical suggestion that troponin, which is closely associated with tropomyosin, is also localized in the thin filament (as we shall see) at regular intervals, each molecule being associated with one troponin complex. The triggering step in the activation of muscle is the combination of Ca^{2+} with one of the troponin subunits, viz., troponin C. In view of the localization of the Ca^{2+} receptor in the thin filaments, the system prevalent in vertebrate striated muscle is referred to as the regulated thin filament. This contrasts with the thick filament regulation, present in a variety of lower organisms, such as molluscs, discovered by Szent-Gyorgyi and colleagues,[17-19] where Ca^{2+} binds directly to myosin, and with the system in smooth muscle, where according to the majority of workers in the area, Ca^{2+} regulation occurs through the activation of a kinase catalyzing the phosphorylation of a component of myosin (although the participation of additional factors can at present not be excluded). We shall not deal with these thick filament or myosin-regulated systems and the reader is referred to several recent reviews.[20-27]

Since the early 1970s the generally accepted view concerning the mechanism by which the interaction of Ca^{2+} with the thin filament-bound regulatory system controls the actin and myosin system has been that in the relaxed muscle, actin and myosin are dissociated. Upon the binding of Ca^{2+} to troponin, a chain of events is initiated which results in the binding of myosin to actin. It has also become very generally accepted, on the basis of X-ray diffraction and electron microscopic investigations to be discussed in detail in this review, that the activation process is accompanied by movement of tropomyosin on the actin filament; a detailed scheme has evolved that can be subsumed under the term steric blocking. According to this view, myosin competes with tropomyosin for binding sites on actin; thus inhibition of actin-myosin interaction is the result of direct blocking by tropomyosin. It has, however, been clear from the beginning that in its purest form this view is untenable since, as we shall see, early evidence existed showing that in the presence of Ca^{2+}, the actin-myosin system containing the regulatory proteins behaves differently from the pure actin-myosin system, which suggests additional changes than simply the removal of steric blocking.

More recently, both biochemical and structural evidence has been put forward, posing a serious challenge to the steric blocking model. These problems, together with evidence bearing on various cooperative phenomena among tropomyosin units and the associated actin monomers, will be discussed. The most radical challenge to the views developing since the Cold Spring Harbor Symposium of 1972, which seemed to witness an essential understanding of the structural and biochemical basis of regulation in striated muscle, comes from recent data suggesting that not only is the problem of steric blocking moot, but that the essence of regulation lies not in the control of the binding of myosin to actin but rather in changes in certain crucial kinetic transitions of actin-bound myosin. Clearly the last word has not been pronounced on these problems and we hope the evidence presented in this review will help the reader to obtain a reasonably clear view of the present, often unclear, situation and help in reaching their eventual clarification.

The reader is referred to a number of other recent reviews that touch upon topics presented here. These include articles dealing with the structure and function of troponin[28,29] and other related calcium binding proteins,[29-33] as well as actin[34,35] and tropomyosin.[36,37] Other articles address the problem of thin filament regulation[38,39] and the current challenges to the steric blocking model.[40-43] Finally, the nature of the actin-myosin-ATP interaction is reviewed by several groups.[7,8,44]

II. TROPONIN

Ebashi and Kodama[11,12] showed that the calcium requirement for ATPase activity of actomyosin depended on the presence of tropomyosin and another protein factor,

Table 1
PROPERTIES OF TROPONIN SUBUNITS

Subunit	Mol wt[a]	Characteristics[60]
TnC	17,846[78]	Ca^{2+} binding
TnT	30,503[211]	Tropomyosin binding
TnI	20,897[189]	Inhibition of actomyosin ATPase activity

Note: Superscript numbers refer to references.

[a] Calculated from the amino acid sequences.

which they named troponin. It was subsequently shown that troponin binds calcium[45-48] and, attached to tropomyosin, is part of the thin filament.[49] Early studies on the electrophoretic behavior of troponin led to the conclusion that it was a single homogeneous protein[47,50] and reported molecular weights ranged from 35,000 to 80,000.[14,46,51,52]

A. Subunits

The first evidence that troponin consisted of at least two proteins was put forward by Hartshorne and Mueller.[53] They obtained two fractions, troponin A and troponin B, both of which were required to confer calcium sensitivity on actomyosin in the presence of tropomyosin. Schaub and Perry[52] and Schaub et al.[54] established that one of the two fractions inhibited actin activation of ATP hydrolysis by myosin, while the other, named "EGTA sensitizing factor", was required for calcium control of ATP hydrolysis. Various other workers at that time separated troponin into two to four fractions[55-58] using gel electrophoresis and ion-exchange chromatographic techniques.

The differences in the numbers and properties of troponin components isolated in various laboratories were largely resolved in two papers by Greaser and Gergely.[59,60] They reported separation of four fractions using DEAE-Sephadex chromatography in 6 *M* urea with approximate M_r of 14,000, 21,000, 24,000, and 35,000 on SDS gels. Reconstitution experiments indicated that the three larger components were involved in modulating actomyosin ATPase activity, while the 14,000-Da component was considered a product of proteolysis. A study of the physical and physiological properties of the three essential subunits led to the currently accepted nomenclature in which the last letter in each abbreviation relates to a specific subunit property. Thus, TnC is the Ca^{2+}-binding subunit; TnT, the tropomyosin, binding subunit, serves as a link to attach the troponin complex to the thin filament via tropomyosin; and TnI, the inhibitory subunit, by itself prevents ATPase activity in a system containing tropomyosin and actomyosin (Table 1). It should be noted that the precise relation of the inhibition of actomyosin ATPase by TnI alone to its role in the inhibition of the complete system in the absence of Ca^{2+} has not been firmly established; this point will be further discussed below. Troponin B of Hartshorne and Mueller[53] and the inhibitory factor of Schaub and Perry[52] were shown to be binary complexes of TnI and TnT, whereas troponin A[53] or EGTA sensitizing factor[52,54] is essentially identical to TnC.

Early studies involving reconstitution of thin filaments from isolated components[59,61-63] and quantitative analysis on polyacrylamide gels of myofibrillar preparations[64] support the view that troponin consists of one molecule each of TnC, TnI, and TnT. This subunit stoichiometry has, however, not gone unchallenged. Recently, Sperling et al.,[65] using a novel preparative method, reported the isolation of a troponin

complex with the subunit composition TnT-TnI_2-TnC on the basis of electrophoresis, analysis of the cysteine content, and ultracentrifugation. The failure of previous preparations to yield this composition, they argue, results from selective proteolysis of one of the TnI moieties. It is, however, difficult to envision this in light of the variety of techniques currently employed to isolate troponin, a number of which include steps specifically designed to remove or inactivate proteolytic enzymes.[57,66,67]

B. Troponin C

TnC, first separated from the troponin complex as troponin A,[53] was identified as the Ca^{2+} receptor moiety. In a number of closely following reports, TnC was shown to be a highly acidic protein (pI = 4.1 to 4.4[66]) containing a large number of aspartic and glutamic acid residues.[54,55,58,60] Its molecular weight was placed at 17,000 to 24,000 with values of 18,300 to 18,500 emerging as the most reliable based on high-speed analytical ultracentrifugation[55,56] and SDS polyacrylamide gel electrophoresis.[60] Other properties of TnC noted in earlier studies were (1) the unusually low A_{280}/A_{260} ratio,[55,60] ascribable to the high phenylalanine to tyrosine ratio coupled with the absence of tryptophan;[60] (2) the presence of about 30% α-helical content as determined by circular dichroism;[53] (3) the heat stability;[53] (4) the ability of the protein to refold after denaturation in 6 *M* guanidine hydrochloride;[53] and (5) the ability of TnC to solubilize the other troponin subunits in low ionic strength media.[60] Although TnC was originally believed to remain monodisperse in solutions varying widely in ionic strength owing to its high net charge at neutral pH, it has been recently shown to form dimers[68] in the presence of millimolar Ca^{2+}.

1. Calcium Binding

Early studies on the Ca^{2+}-binding properties of TnC were hampered by the lack of well-defined protein preparations and by the technical difficulties in controlling the free Ca^{2+} levels. Without the use of metal buffers, sufficiently low Ca^{2+} concentrations could not be realized, presumably because of contaminating Ca^{2+}. The high affinity sites of TnC went undetected in studies involving equilibrium dialysis and ultrafiltration.[69] Similar problems were encountered in work using ion exchange resins to control Ca^{2+} levels.[46,48,55,60,70] The use of Scatchard plots[71] gave rise to problems in data analysis leading to a wide range of numbers and affinities of Ca^{2+}-binding sites in the earlier literature,[46,48,55,60,69,70,72] viz., one to five sites with affinity constants of 10^6 to 10^4 M^{-1}.

Potter and Gergely[73] incorporated Ca^{2+}-EDTA or Ca^{2+}-EGTA buffer systems into their equilibrium dialysis medium and used a computerized nonlinear least squares curve-fitting procedure[74,75] instead of the Scatchard plot. Assuming several classes of independent binding sites, they obtained the best fit to their data with four Ca^{2+}-binding sites falling into two classes: one class containing two high affinity sites that also bind Mg^{2+} competitively, referred to as the Ca^{2+}-Mg^{2+} sites, and the other containing two lower affinity sites that selectively bind Ca^{2+}, the Ca^{2+}-specific sites. In addition, two other Mg^{2+}-specific sites were suggested by this work; however, further indication of their existence has proved elusive with the exception of a single recent paper by Sperling et al.[65] The properties of the metal binding sites in Tn are summarized in Table 2.

2. Parvalbumins as Structural Models for TnC

The amino acid sequence of rabbit skeletal TnC[77,78] reveals that it consists of a single polypeptide chain of 159 residues. Its calculated molecular weight is 17,846 and net charge at neutral pH is −30, assuming that all carboxyl, amino, guanidino, and imidazole groups are dissociated. There are no tryptophan residues, only one each of cysteine (#98), proline (#50) and histidine (#135), and two each of asparagine (#150, #142)

Table 2
BINDING CONSTANTS OF CALCIUM AND MAGNESIUM TO TnC

Sites	K_{Ca} (M^{-1})	K_{Mg} (M^{-1})	Ref.
Ca^{2+}-Mg^{2+}	2×10^7	5×10^3	73
	$2 \times 10^{6.a}$		73
Ca^{2+}-specific	5×10^5		73
		2×10^2	76
Mg^{2+}-specific		5×10^3	73

[a] In the presence of 2 mM $MgCl_2$.

and tyrosine (#10, #109). Perhaps most important were the observations of Collins et al.[77] and Collins,[79] who recognized four homologous regions in the sequence, each of which presumably contained one of the four Ca^{2+}-binding sites of Potter and Gergely,[73] which were also homologous to similar regions previously described in another group of intracellular Ca^{2+}-binding proteins, the parvalbumins.

Although known for many years to exist in the muscle of certain fish and amphibians,[80] the parvalbumins were first characterized by the chemical and physicochemical studies of Hamoir et al.[81-83] and Pechere et al.,[84-86] who established their evolutionary relatedness and common physical properties on the basis of extensive studies carried out on parvalbumins isolated from a wide variety of species. Parvalbumins are typically low molecular weight (~12,000), acidic (pI = 4.0 to 4.5), water-soluble peptides. In 1971-72, studies conducted by Pechere and colleagues[87,88] and by Nockolds et al.[89] established that parvalbumins from hake and carp muscle each bind 2 mol of Ca^{2+} per mole protein ($K \sim 2 \times 10^7$ M^{-1}), which led to their designation as muscle calcium binding proteins (MCBPs). The determination of the amino acid sequences from hake[90] and carp[91] parvalbumins and the X-ray diffraction studies on crystals of the latter, leading to their three-dimensional structure,[92,93] have contributed significantly to our understanding of TnC.[79]

In carp parvalbumin, the 108-residue polypeptide chain is folded into 6 α-helical segments of about 8 to 12 residues each, designated A to F, starting from the amino terminus. Between each pair of helices lies a stretch of 12 residues that form a reverse turn or loop. The loops between helices C and D and between E and F each bind a Ca^{2+} ion. The Ca^{2+}-binding loops are rich in carboxylate-containing amino acid side chains (e.g., Asp 51 and 53 and Glu 59 and 62 in the CD loop; Asp 90, 92, and 94 and Glu 101 in the EF loop) that contain ligands for coordination with Ca^{2+} ions.[92] The AB region of the molecule, although it exhibits the same helix-loop-helix arrangement as the CD and EF regions, does not bind Ca^{2+} owing to the lack of sufficient ligands in its loop.

The spatial arrangement of the helix-loop-helix unit was described by Kretsinger and Nockolds[92] as being similar to a right hand whose thumb and forefinger, extended at approximately right angles to each other, point toward the COOH-terminus of helix F and the NH_2-terminus of helix E, respectively. The middle finger, curled towards the base of the thumb, traces the course of the Ca^{2+}-binding loop around the bound ion (Figure 1). The term "EF hand" has become commonly used to describe the structure of Ca^{2+}-binding regions in intracellular metal binding proteins. This is perhaps an unfortunate development insofar as the designation tends to obscure specific differences that exist within the overall helix-loop-helix arrangement of the different sites. Even within carp parvalbumin, a bend in the D helix at residue 65 causes the helix to turn outward, a feature not seen in the homologous E helix. In this paper, we will refer to Ca^{2+}-binding domains implying the α-helix-loop-α-helix arrangement.

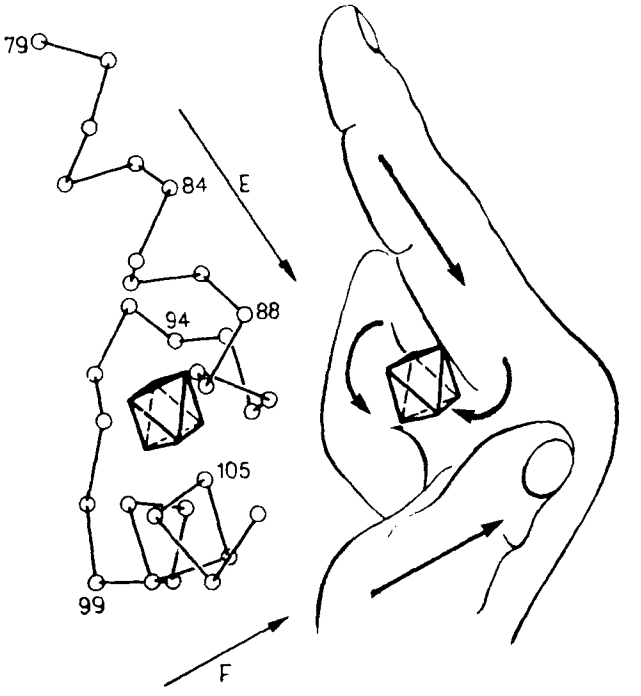

FIGURE 1. Spatial arrangement of the helix-loop-helix of a Ca^{2+}-binding domain represented by the three fingers indicated of a right hand. The left side of the figure depicts the positions of the alpha carbon atoms of the EF loop of carp parvalbumin taken from the X-ray structure.[92] (Reproduced, with permission, from the *Annual Review of Biochem.*, Volume 45, © 1976 by Annual Reviews Inc.)

With the availability of the above information on parvalbumin and the sequence of TnC, Collins and colleagues[77] and Kretsinger and Barry[94] described the latter as consisting of four Ca^{2+}-binding domains, numbered I to IV, starting from the NH_2-terminus of the molecule. Each domain contained residues that, by their homology to parvalbumin, suggested the same helix-loop-helix arrangement and thus, a similar overall three-dimensional structure. The best alignment (i.e., that giving the largest number of amino acid identities and conservative replacements) of the two proteins occurred when the AB, CD, and EF domains of carp parvalbumin were positioned opposite domains II, III, and IV of TnC (Figure 2).

3. Three-Dimensional Structure

There is no direct X-ray crystallographic support for models of TnC structure owing to the difficulty of obtaining stable crystals that are large enough to obtain high resolution patterns. Crystals of rabbit skeletal TnC prepared in the presence of $MnCl_2$[95] were not sufficiently stable when exposed to X-rays to allow resolution beyond 8 to 10 Å. Recently, Strasburg et al.[96] obtained stable crystals of TnC from chicken skeletal muscle which yielded good diffraction spots out to about 2 Å and thus seemed suitable for high resolution work. Until results on these crystals are available, the best model of TnC remains that proposed by Kretsinger and Barry[94] on the basis of TnC-parvalbumin homologies and the fundamental assumption that homologous primary structures yield superimposable tertiary folds. With the constraints of packing and connectivity, their model considers domains I and II and III and IV to constitute two parvalbumin-like halves, their twofold axes being coincident and antiparallel. The best

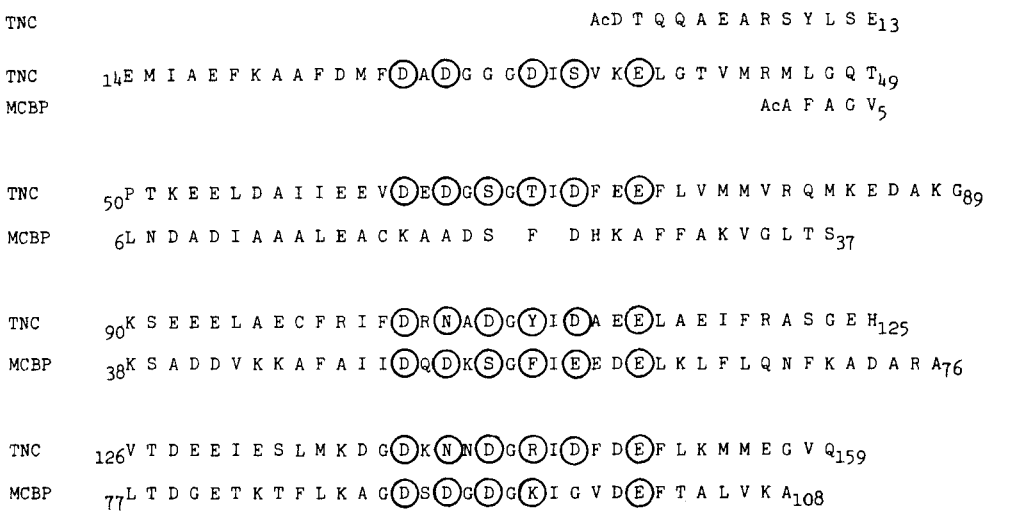

FIGURE 2. Amino acid sequences of rabbit skeletal TnC and carp parvalbumin (MCBP). The homology of the sequences is particularly clear in the metal-binding regions. The circled residues act as metal binding ligands.[79,91] Amino acid code: A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile: K = Lys; L = Leu; M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Thr; V = Val; W = Trp; Y = Tyr.

fit was obtained with a separation of 35 Å and a rotation by 20° of one half with respect to the other about the common axis. Figure 3 schematically illustrates the features of the model. In it, TnC appears as a prolate ellipsoid of revolution in which four Ca^{2+}-binding loops are open to the surface of the molecule. Loops I and II and loops III and IV constitute two pairs at either end of the long axis of the molecule. The two sites in the NH_2-terminal half have been identified as the low affinity, Ca^{2+}-specific sites; those in the COOH-terminal half are the Ca^{2+}-Mg^{2+}sites (see Section II). Ca^{2+} ions bound to sites within the same class are separated from each other by about 11 Å while those bound to different classes are about 33 Å apart. Among other features that emerge from the Kretsinger and Barry model are that Tyr 109, located in the sequence of loop II, is also close (<5 Å) to loop IV and may serve as a fluorescent reporter group of metal binding to either site and that the thiol group of Cys 98 is partially buried. Both of these predicted features have since been confirmed by experiments on the protein in solution (see below).

4. Properties of the Metal Binding Loop

The binding of Ca^{2+} or Mg^{2+} to a particular loop in TnC or its homologs depends on how well the loop conforms to the coordination geometry and chemistry of the ion. Calcium and Mg^{2+} have ionic radii of 0.99 and 0.64 Å, respectively. In aqueous solution, Mg^{2+} maintains a closely regular octahedral hexacoordinated state. Mg^{2+}-O bond angles and distances tend to be invariant, the latter ranging from 2.00 to 2.12 Å in a variety of simple ligands and crystals (compare Reference 97). In the case of Ca^{2+}, water molecules, while also octahedrally oriented (C.N. 6),[98] are considerably more mobile and the ion can shift easily to coordination numbers of 7 and 8. Bond angles vary widely as do Ca^{2+}-O distances which range from 2.30 to 2.82 Å with an average of 2.40 Å.[98] Thus Mg^{2+} requires a specific geometry of its ligands for tight binding, whereas Ca^{2+} is perhaps better able to conform to the irregular geometries of coordination sites in biological molecules. Not only do the two ions differ in coordination geometry, but also with respect to the nature of the coordinating atom: Ca^{2+} ions prefer

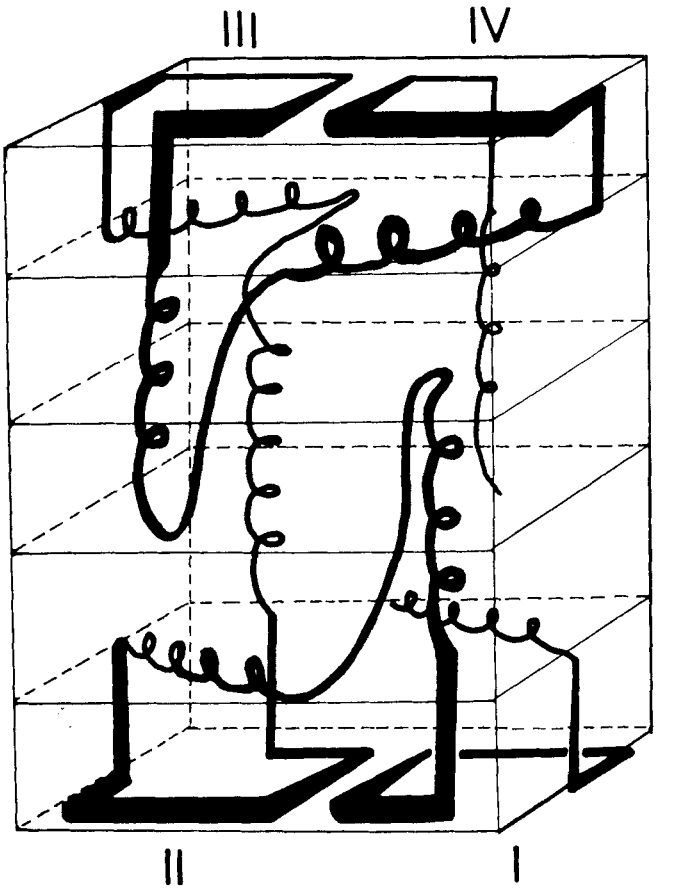

FIGURE 3. Schematic diagram of TnC structure showing the four Ca^{2+}-binding loops (I to IV) and the flanking helices.[94] Note that an NH_2-terminal segment, residues 1 to 14, for which there is no homologous sequence in parvalbumin, is not shown. (From Gergely, J. and Leavis, P. C., *Muscle Contraction: Its Regulatory Mechanisms*, Ebashi, S., Maruyama, K., and Endo, M., Eds., Springer-Verlag, Berlin, 1980, 191. With permission.)

oxygen atoms, Mg^{2+} nitrogen. In proteins that tightly bind Ca^{2+}, generally three or more of the ligands are derived from carboxylate groups, the others being donated by carbonyl groups of the peptide backbone or ether groups of the side chains.

The metal binding sites of parvalbumin and TnC appear precisely tailored to accommodate the Ca^{2+} ion with high affinity. Kretsinger and Nockolds[92] have shown that in parvalbumin, the Ca^{2+} ions are coordinated by six oxygen ligands arranged octahedrally. They designated the six ligands as $\pm X$, $\pm Y$, and $\pm Z$, corresponding to the axes of a local coordinate system in which the origin is at the center of the ion. Comparison of the sequences of the two binding loops of parvalbumin with those of the four TnC loops, as well as a variety of loops from other intracellular Ca^{2+}-binding proteins, reveals remarkable similarities (Table 3).[30,79,99] First, the loops are typically 12 amino acid residues long and the coordinating ligands are contributed by residues occupying positions 1, 3, 5, 7, 9, and 12, starting from the NH_2-terminus of the loop. In some cases, when a suitable oxygen ligand does not occur at the appropriate coordinate position, it is instead donated by a nearby ligand in the loop (e.g., each of the two oxygen atoms in the carboxylate groups of Asp 92 and Glu 101 in MCBP forms one of

Table 3
PRIMARY STRUCTURES OF HOMOLOGOUS CALCIUM BINDING PROTEINS — COMPARISON OF THE AMINO ACID SEQUENCES OF THE CALCIUM BINDING LOOPS[99]

Protein	Domain	Sequence[a]	Amino acids
Paravalbumin (carp muscle)	AB	K A A D S F D H K A	19—28
	CD	D Q D K S G F I E E D E	51—62
	EF	D S D G D G K I G V D E	90—101
TnC (rabbit skeletal muscle)	I	D A D G G G D I S V K E	27—38
	II	D E D G S G T I D F E E	63—74
	III	D R N A D G Y I D A E E	103—114
	IV	D K N N D G R I D F D E	139—150
Calmodulin (calf brain)	I	D K D G N G T I T T K E	20—31
	II	D A D G N G T I D F P E	56—67
	III	D K D G N G Y I S A A E	93—104
	IV	N I D G D G E V N Y E E	129—140
Alkali light chain (rabbit skeletal muscle)		D R T G D S K I T L S Q	59—70
		D E Q M N A K I E F E Q	96—108
		D K E D G T V G M G A E	136—147
		Q E D S N G C I N Y E A	171—182
Calcium binding protein (swine intestinal mucosa)		D K N G D G E V S F E E	57—68

[a] Key to one-letter code for amino cids: A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Thr; V = Val; Y = Tyr.

the octahedral vertices around the bound Ca^{2+} [compare Reference 30]) or by one or more water molecules. Virtually all of the loops contain at least four carboxylate ligands, although the number of carboxylate-containing residues in a given loop is not directly correlatable with binding strength.

Although these features, common to the over 50 12-residue loop sequences currently available for comparison, are useful indicators of Ca^{2+} binding per se, it is nonetheless difficult to deduce solely from an examination of a loop sequence either the strength or specificity of its interaction with the ion. Early speculation based on differences in the loop sequence aimed at distinguishing sites of the high and low affinity class led to contradictory results.[94,100] Several recent predictive studies appear, at least, partially successful. Vogt et al.[101] have developed a method based on the frequency distribution of "β-turn forming residues" in the loop. They found that loops that are known to bind Ca^{2+} have a high frequency of β-turn forming residues (compare Chou and Fasman[102]) located in the first and third tetrapeptide of their 12-residue loops relative to loops that do not bind Ca^{2+}. Although the presence of these residues correlates well with Ca^{2+}-binding as such to a particular loop, it does not provide information concerning strength or specificity of the binding. Potter et al.[103] and Kretsinger[104] propose that the presence in the loop of a glycyl residue between the calcium coordinating ligands at positions Y and Z confers a Ca^{2+}-specific lower affinity status in the loop. Finally, an extensive analysis of 45 loops by Reid and Hodges[105] predicts the Ca^{2+} affinity on the basis of the identities of residues in key positions within the 12-residue sequence; viz., positions 7 and 10. According to their findings, the size and orientation

of these residues can modulate the replacement of water molecules in the cation hydration shell subsequent to initial ion-pair formation with a single negatively charged group and prior to chelation by the other coordinating groups in the loop. Reid and Hodges[105] further offer a "two-site cooperative model" in which metal binding to one of a pair of sites induces a conformational change that affects the positions of the side chains of residues 7 and 10 in both loops. This can result either in the destabilization of metal binding to the first site or the enhancement of binding to the second.

Although there is little doubt that differences in the position and identities of key residues within a dodecapeptide loop contribute to its metal-binding properties, the flanking α-helices as well as more distant parts of the molecule are likely to exert an influence. Reid et al.[106,107] prepared synthetic analogs of domain III of TnC in which the loop itself and the α-helix on the COOH-terminal flank of the loop were intact, but the NH_2-terminal α-helix was clipped at various points. Their shortest peptide, comprising residues 103 to 123, was able to bind Ca^{2+}, but with an apparent binding constant of only $3.1 \times 10^2\ M^{-1}$. In contrast, when the peptide was lengthened to include the missing α-helix (residues 90 to 123), the binding constant increased by four orders of magnitude to $5.1 \times 10^6\ M^{-1}$.

Evidence for longer range, viz., site-site, influence on binding properties was first dramatically demonstrated by the studies of Coffee and Solano[108] on parvalbumin. They showed that when the molecule was cleaved between its two Ca^{2+}-binding sites, leaving each domain intact, metal binding was entirely lost. This was attributed to the loss of a single hydrogen bond that stabilized the tertiary fold of the intact protein. Leavis et al.[109] have shown that the two high affinity sites in TnC, when separated into two single-site proteolytic fragments, display only moderate affinities for Ca^{2+} and essentially no Mg^{2+} binding. It is only when the two sites are present in the same peptide that they acquire the "native" high affinity Ca^{2+}-Mg^{2+} binding characteristics.

5. Calcium-Dependent Conformational States of TnC

A number of spectral changes accompany the binding of Ca^{2+} to the Ca^{2+}-Mg^{2+} sites which includes changes in UV absorption,[110-112] intrinsic (tyrosine) fluorescence[113-116] and fluorescence polarization,[113] laser Raman spectrum,[117] circular dichroism,[65,111-114,118-122] and optical rotatory dispersion.[123] The increase in circular dichroism in the 222-nm region indicates an increase in α-helical content from about 30% in the divalent cation-free protein to about 50% on saturation of the Ca^{2+}-Mg^{2+} sites. This corresponds to the formation of two 8- to 10-residue α-helical regions[111] and a general "tightening" of the structure which is supported by hydrodynamic data that show an increase in the $S_{20,w}$ and a decrease in the viscosity of a TnC solution upon addition[122,124] of Ca^{2+} and by the observation that the susceptibility of the protein to tryptic digestion is decreased with Ca^{2+} present.[125] In addition to the far UV changes in circular dichroism, changes also occur in the near UV circular dichroism and absorbance,[111] suggesting an involvement of phenylalanine residues. On the basis of the distribution of phenylalanine residues in the amino acid sequence, one of the helical segments formed has been located on the NH_2-terminal side of site III (Figure 4) and the other on the COOH-terminal side of site IV (helices E and H of Kretsinger and Barry[94]). In the absence of Ca^{2+}, the addition of millimolar concentrations of Mg^{2+} induces qualitatively similar changes in the above spectral properties to those brought about by Ca^{2+}, although only ~40% of the Ca^{2+}-induced fluorescence change and ~85% of the far UV circular dichroism change can be elicited by Mg^{2+}.[114]

Recently, ^{1}H-NMR spectroscopy has been used to study the solution conformation of TnC. In the NMR spectrum of a folded protein, resonance lines arising from various amino acids are shifted from their primary positions (i.e., the positions they would occupy in a configuration in which they are accessible to the solvent). These so-called

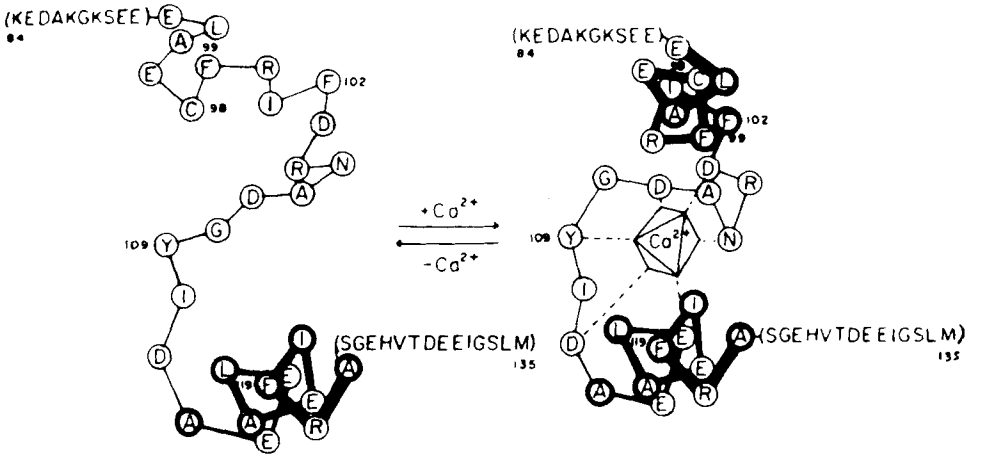

FIGURE 4. Ca^{2+}-induction of helical structure in a stretch of residues (#91 to 102) flanking metal binding site III in troponin C.[442]

secondary shifts depend upon H-bonding, shielding caused by nearby charged groups, the degree of ionization of ionizable groups, and ring current shifts produced by aromatic groups (compare Reference 126). In the case of TnC, secondary shifts in a number of resonances indicate a Ca^{2+}-induced increase in secondary structure in the high affinity regions of the protein. Other changes detected by ^{1}H-NMR include Ca^{2+}-induced broadening of resonances assigned to tyrosine, phenylalanine, glutamate, and aspartate residues, all indicative of increased constraints on their movement.[127-131] Many of these changes have also been observed in tryptic fragments of TnC containing only the two Ca^{2+}-Mg^{2+} sites.[132,133]

Ca^{2+}-induced changes in the COOH-terminal half of TnC are also reflected by the behavior of various optical and spin probes attached to Cys 98, located within the NH_2-terminal helix of region III,[109,134-138] and to other residues in the molecule.[112,139,140] Furthermore, the reactivity of Cys 98 to various thiol reagents is reduced by Ca^{2+}.[134] Other residues showing Ca^{2+}-dependent environmental changes are Lys 136 and 140, in which reactivity to acetylation is decreased by Ca^{2+};[141] His 125, in which pK is decreased;[127-129] and Tyr 109, which shows a change in interactions involving its phenolic hydroxyl group.[115,117]

In contrast to the large changes brought about by Ca^{2+} binding to the sites in the high affinity class, binding to the Ca^{2+}-specific sites is generally believed to produce more subtle structural perturbations.[111,127,128,130] The circular dichroism analysis of Nagy and Gergely[111] concluded that the observed far UV changes may be interpreted as an addition of a peptide unit or two to one or more of the preexisting helical segments in the NH_2-terminal half of the molecule. According to two other reports,[119,120] up to 40% of the secondary change in the whole molecule results from Ca^{2+} binding to the low affinity sites in the pCa range of 6 to 5. The latter observations are difficult to reconcile with experiments at higher protein concentrations in which spectral changes are essentially complete on addition of 2 mol of Ca^{2+}/mol[121] and with results from circular dichroism studies on TnC and its proteolytic fragments,[109] laser Raman spectroscopy,[117] and ^{1}H-NMR spectroscopy.[127,128,142] All of these studies are consistent with relatively small ($\sim$10%) changes in secondary structure.

Changes occur in the tertiary structure upon occupation of the Ca^{2+}-specific sites in addition to whatever secondary structural changes occur. While ^{1}H-NMR studies provide little clear-cut evidence of backbone changes, broadening of several resonances corresponding to hydrophobic side chains of leucine, isoleucine, and phenylalanine

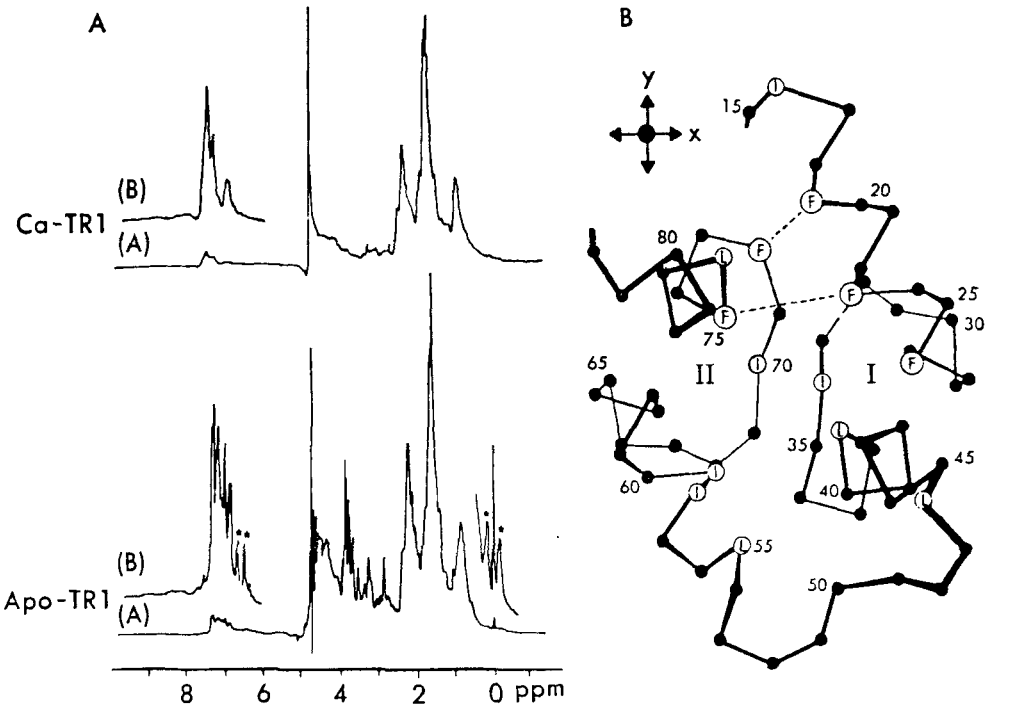

FIGURE 5. A: (A) ^{1}H-NMR spectra of the tryptic fragment TR1C of TnC (residues 9 to 84) in the absence (apo-TR1) and presence of Ca^{2+} (Ca-TR1). (B) Resolution-enhanced spectra of aromatic resonances and upfield methyl signals. In the spectrum of apo-TR1, the stars indicate the positions of the two ring-current-shifted phenylalanine signals (δ = 6.64 and 6.42 ppm) and the two upfield methyl signals corresponding to Ile (δ = 0.19 ppm) and Leu (δ = −0.16 ppm).[142] These shifted resonances all disappear upon addition of Ca^{2+}. B: Schematic representation of the conformation of regions I and II of TnC involving helix-helix contacts between the NH_2-terminal helix of region I and the COOH-terminal helix of region II in the absence of Ca^{2+}. The structure is represented as two regions comprising a stretch of approximately 30 residues, each in the helix-loop-helix configuration by analogy with parvalbumin.[94] (From Gergely, J. and Leavis, P. C., in *Muscle Contraction: Its Regulatory Mechanisms*, Ebashi, S., Maruyama, K., and Endo, M., Eds., Springer-Verlag, Berlin, 1980, 191. With permission.)

residues suggests local changes in their environment attributable to changes in the tertiary fold. In particular, the disappearance of ring-current-shifted resonances arising from one or more phenylalanine residues upon the addition of the third and fourth Ca^{2+} to TnC suggests the loss of hydrophobic contacts between pairs of phenylalanines.[127,128]

Recently fresh light has been shed on this process by studies on TnC fragments containing only the Ca^{2+}-specific binding domains and including only five of the ten phenylalanine residues of the parent protein (TR1C, residues 9 to 84 and TH1, residues 1 to 120[142]). The hydrophobic side chain signals in the spectra of the fragments are the same as in intact TnC, suggesting that the local environments of the relevant side chains are retained in the fragments. A more detailed analysis of the upfield ring-current shifted Phe resonance (δ = 6.64 and 6.42 ppm) suggests that in the apoprotein, two pairs of phenylalanine residues, separated by some 50 residues in the primary structure, interact to stabilize the tertiary folds of the peptide. On the basis of the tentative coordinates proposed by Kretsinger and Barry,[94] the most likely mutually stabilizing pairs are 19 to 72 and 23 to 75 (Figure 5). Binding of Ca^{2+} to sites I and II results in a loss of these contacts as well as in changes of the environments of leucine and isoleucine residues in which interactions probably also contribute to the stability

of the apoprotein. All the changes are consistent with the bending or sliding of helices A and D relative to one another.[142]

The following picture of the overall structure of TnC emerges from the studies described above. In the apoprotein, domains I and II, constituting the NH_2-terminal half of the molecule, are highly organized with preformed α-helical segments, each containing eight to ten residues flanking each of the two sites. This structure is stabilized by hydrophobic contacts involving phenylalanine, leucine, and isoleucine residues. In contrast, in domains III and IV, constituting the COOH-terminal half, only two of the four potential helices (F and G) are actually coiled, while the others exist in an extended configuration. Binding of the first two Ca^{2+} ions to sites III and IV induces the coiling of helices E and H,[111] which would account for ~90% of the observed increase in secondary structure induced in the molecule by Ca^{2+} and the concomitant formation of stabilizing Phe-Phe linkages in the COOH-terminal half of the molecule, which stabilize the new Ca_2-TnC conformation.[132] These hydrophobic contacts appear to involve phenylalanine residues located in helices E and H.[133] In addition, the COOH-terminal half of Ca_2-TnC appears to be stabilized by an as yet unspecified salt bridge.[132] It is interesting to note that the protein in this form, which is likely to be the form in which it exists in relaxed muscle (viz., either Ca_2- or Mg_2-TnC), is highly symmetrical insofar as both halves have a high degree of secondary structure stabilized by hydrophobic bonds arising from side chains in homologous positions. The subsequent binding of Ca^{2+} to regions I and II results in the loss of the NH_2-terminal hydrophobic contacts leading to a tertiary structural change in this region, possibly accompanied by the addition of one or two turns to, or a tightening of, the preexisting helices (compare References 111 and 142).

6. Assignment of High and Low Affinity Sites in Troponin C

The first experimental clues as to which of the four sites in TnC are the high affinity and which are the low affinity sites came from studies employing optical and spin probes attached to Cys 98, which is located within domain III. The fact that the spectral properties of these probes were sensitive to the binding to the protein of the first two metal ions suggested that site III belonged to the Ca^{2+}-Mg^{2+} class.[134] The identification of site I as a low affinity Ca^{2+}-specific site is suggested by binding studies on cardiac TnC which reveal two high affinity Ca^{2+}-Mg^{2+} sites, but only a single low affinity site.[103,139,143,144] A comparison of the amino acid sequences of cardiac and skeletal TnC shows only conservative replacements in all regions of the molecule save the loop of domain I. Amino acid substitutions involving key coordinating ligands suggest that in cardiac TnC[145] and TnC from slow muscle,[146] site I is nonfunctional (Figure 6).

More direct assignments of the regions have come from Ca^{2+}-binding studies using proteolytic fragments of TnC produced by digestion with CNBr, trypsin, and thrombin[109] and by chemical modification of carboxylate-containing side chains.[147] Both studies have established that the Ca^{2+}-specific sites are in domains I and II while the Ca^{2+}-Mg^{2+} sites are in domains III and IV.

7. Kinetics

Information on the two classes of metal binding sites in TnC obtained from equilibrium binding studies has been usefully supplemented by kinetic studies. These have been done chiefly by means of stopped-flow fluorescence spectroscopy, but kinetic information is also derived from NMR spectra.

Stopped-flow studies depend on the availability of suitable intrinsic or extrinsic chromophores that report either the combination of the metal with the protein or the subsequent structural rearrangement, or both. Thus, the rate of an optical change observed on mixing Ca^{2+} or Mg^{2+} with TnC or those observed on the removal of the ions

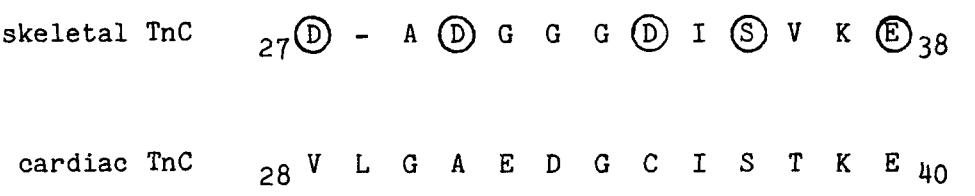

FIGURE 6. Comparison of the amino acid sequences in Ca^{2+}-binding loop I of bovine cardiac and rabbit skeletal TnC.[145] Circled residues in skeletal TnC are the metal binding ligands.

by chelation do not necessarily represent the rate of the combination of the metal with the protein or its dissociation, respectively. They may represent reactions that follow metal binding or dissociation; hence, their relation to the measured equilibrium constants is not always simple. In general, the following scheme applies:

$$M + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} MP^*$$

The equilibrium binding constant is given by $K_b = k_1k_2/k_{-1}k_{-2}$. If the conformational changes associated with the second step are rate limiting, the measured rate constants would be k_2 and k_{-2}. NMR exchange measurements also provide information about k_2 and k_{-2}.

Stopped-flow spectroscopic measurements have utilized Tyr 109 of skeletal TnC as an intrinsic fluorophor[148-150] as well as various reagents (BIPM, ANM, and DACM)[135-137] attached to Cys 98 as reporters of binding at the high affinity sites. Dansylaziridine (DANZ)[151] has been reported as being preferentially attached to Met 25 and as a reporter of changes at the low affinity sites, although some changes occurring at the high affinity sites are also reflected in its spectral behavior (compare Table 4). Experiments with the luminescent metal Tb^{3+} provide direct information about its binding and dissociation at the high affinity sites.[121]

For the removal of Ca^{2+} by EGTA from the high affinity sites, utilizing the fluorescence of tyrosine, a rate constant of 1.3 sec^{-1} has been obtained.[148-150] This value is in good agreement with the rate constant obtained from the small increase in the fluorescence of DANZ-TnC[152] and with that derived from the exchange of Tb^{3+} with Ca^{2+}.[121] The fact that changes are found in the spectrum of the DANZ fluorophor attached to a site in an NH_2-terminal domain under conditions when Ca^{2+} is being removed from the high affinity sites in the COOH-terminal half of the molecule indicates that structural changes in one half of the molecule affect the other half. Whether or not these rates represent the actual rate of dissociation or whether they represent a rate-limiting conformational change — which, however, appears unlikely in the light of NMR results[127,128,153] — the important point emphasized by Johnson et al.[152] and by Robertson et al.[154] is that the rate of removal from these sites would be too slow to assign to them a control role in the regulation of muscle contraction which occurs on a time scale of milliseconds. This conclusion is in agreement with the previously proposed view that the low affinity sites are the regulatory ones. Iio and Kondo[137] report a rate constant of 3.7-sec^{-1} for the process involving Ca^{2+} removal from the high affinity sites when the reporter fluorophor is an analinonaphthalene label (ANM) attached to Cys 98.

The combination of metal ions with a protein is a second-order process and a detailed study at various free metal ion concentrations would be required in order to evaluate the second-order rate constant. Under the conditions that experiments of this nature are usually carried out, Ca^{2+} and EGTA are first premixed to give the desired

Table 4
RATE CONSTANTS FOR THE BINDING AND RELEASE OF CALCIUM AND MAGNESIUM FROM BINDING SITES ON TnC[152]

Ion	Sites	k_{on} (sec^{-1})	k_{off} (sec^{-1})
Ca^{2+}	Ca^{2+}-Mg^{2+}	>350	1
Mg^{2+}	Ca^{2+}-Mg^{2+}	99—115	8
Ca^{2+}	Ca^{2+}-specific	>350	230—350

free Ca^{2+} and then added to the protein in the stopped-flow apparatus. Even if the final Ca^{2+} concentration is such that only combination at the high affinity sites is intended, owing to the initially higher Ca^{2+} concentration, transient combination at the low affinity sites of TnC may occur and complicate the interpretation of results. This was the case in the work of both Johnson et al.[152] and Iio and Kondo[137] when the DANZ label was used to monitor the binding of Ca^{2+} at a final pCa of about 6 to the high affinity sites. There was a rapid increase — within the mixing time of the instrument — of fluorescence resulting from binding to the low affinity sites followed by a decrease at a rate of ~230 sec^{-1}, indicating dissociation from these sites as the final pCa is approached. Iio and Kondo[136] also showed a slow, 23 sec^{-1}, rate process attributable to a conformational change. In the light of the results with tyrosine fluorescence,[137] the 23 sec^{-1} rate is likely to be the result of Ca^{2+} binding to the high affinity sites. The on-rate for the process monitored by ANM fluorescence has been found to be characterized by a constant of 157 sec^{-1} at pCa = 5.89. This process is followed by a lower rate (6 sec^{-1}) that may be due to a slower conformational change. The combination of Ca^{2+} with the low affinity sites, monitored by DANZ, occurs rapidly within the mixing time of currently available instruments. These high rates (>230 sec^{-1}) are consistent with diffusion-controlled processes at Ca^{2+} concentrations of 10^{-6} *M*.

The on-rate monitored with tyrosine fluorescence is characterized by a constant of 23 sec^{-1}, suggesting a slow conformational change following Ca^{2+} binding to the high affinity sites which, as indicated by the rate reported using ANM, would occur almost ten times faster.

Experiments carried out in the presence of Mg^{2+} are somewhat harder to interpret. Johnson et al.[152] reported an off-rate of 8 sec^{-1} for Mg^{2+} which would limit the binding of Ca^{2+} to the Ca^{2+}-Mg^{2+} sites previously occupied by Mg^{2+}. Iio and Kondo[137] observed a biphasic reaction on combination of Ca^{2+} with what they considered the low affinity sites monitored by DANZ. The first phase was within the dead time of the instrument; the second phase may well have been due to the binding of Ca^{2+} to the high affinity sites limited by the dissociation of Mg^{2+}, rather than a conformational change induced by binding to the low affinity sites. It is somewhat puzzling that Iio and Kondo[137] also report a biphasic reaction for the removal of Ca^{2+} from the low affinity sites when Mg^{2+} is at the high affinity sites. The change is small, but if it is confirmed by other workers, it would suggest that TnC containing Mg^{2+} at the high affinity sites undergoes a secondary change upon removal of Ca^{2+} from the Ca^{2+}-specific sites. It is equally puzzling that the Ca^{2+}-binding rate to the high affinity sites monitored by ANM fluorescence is describable in terms of two processes, one characterized by an apparent first-order rate constant of about 26 sec^{-1} followed by a slower step with a constant of 1.65 sec^{-1}. If the high affinity sites were indeed occupied by Mg^{2+}, then the fastest process observed should not be faster than the Mg^{2+} off-rate. Iio and Kondo[137] state that there is an exchange process involving Ca^{2+} and Mg^{2+} which occurs at a high rate so that it is complete within the instrumental lag time. This too is hard to understand if the reported Mg^{2+} off-rate is of the order of less than 10 sec^{-1}.

Kinetic measurements of metal-protein interactions in the troponin system are clearly of great interest since they provide the link between the molecular, biochemical, and physiological processes. To date, the kinetic studies provide the strongest evidence implicating the low affinity sites as the triggers of contraction. As mentioned above, the rate of Ca^{2+} binding to the high affinity sites under conditions prevailing in muscle would be limited by the relatively slow dissociation of Mg^{2+} from these sites; its rate of release is far too slow in comparison to the tension decay in relaxing muscle. On the other hand, Ca^{2+} binding to, and release from, the Ca^{2+}-specific sites can take place on a time scale consistent with activation and relaxation in vivo.

8. Binding of Ions other than Calcium and Magnesium

Calcium bound to TnC per se or to TnC forming a complex with the other subunits exchanges, at least partially, with a number of mono-, di-, and trivalent cations. Fuchs[155] first showed that Ca^{2+} could be partially displaced from troponin by divalent cations, the effectiveness in decreasing order being $Cd^{2+} > Sr^{2+} > Pb^{2+} > Mn^{2+}$. Under the experimental conditions, no exchange was seen with Mg^{2+}, Ba^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+}, although it has since been shown that this second set of cations can bind to the protein in the absence of Ca^{2+}.[122,156] The earlier work indicated that those ions most successful in competing with Ca^{2+} for protein binding sites had about the same ionic radius as Ca^{2+}; viz., ~1 Å. The radii of the ions in the above list that are capable of displacing Ca^{2+} range from 0.8 Å for Mn^{2+} to 1.2 Å for Pb^{2+}.

Only two monovalent ions, H^+ and Na^+, have been reported to bind to TnC. Lehrer and Leavis[157] showed that H^+ ions induce changes in the spectral properties of the protein similar to those induced by Ca^{2+} with a $K^{APP} = 10^6\ M^{-1}$. This was interpreted as being due to proton binding to carboxylate-containing amino acid residues in the metal binding sites that, because of their close grouping in the loop, possess abnormally high pK values. A subsequent report by Robertson et al.[158] suggests that H^+ competes with calcium at the Ca^{2+} -specific, but not the Ca^{2+}-Mg^{2+} sites, reflecting the different values of K_{Ca}/K_H for the two classes of sites. Sodium has also been shown to bind weakly to TnC[159] both at the high affinity and low affinity sites.

The trivalent lanthanide ions have been widely used as Ca^{2+} substitutes in parvalbumin and TnC owing to their calcium-like preference for octahedral coordination with oxygen atoms and their similar ionic radii.[160] Furthermore, the fact that a number of the lanthanides are either luminescent or paramagnetic makes them attractive as reporter groups of the environments of metal binding sites on proteins. It was shown in the mid 1970s[151,162] that Tb^{3+} substituted for the EF but not the CD calcium in carp paravalbumin. In subsequent reports, however, the ability of the CD site to also bind Tb^{3+} has been demonstrated, although the exchange rate with Ca^{2+} was considerably slower than at the EF site.[163]

In the case of TnC, several early reports[164,165] suggested that one of the two Ca^{2+} ions bound to the high affinity sites was preferentially replaced by Tb^{3+}. However, Leavis et al.[121] subsequently demonstrated that Tb^{3+} exhibited the same preferential binding as does Ca^{2+} for the two Ca^{2+}-Mg^{2+} sites, as judged from fluorescence titrations in which the Tb^{3+} was excited via Tyr 109 of the protein. Furthermore, Tb^{3+} readily displaces both high affinity Ca^{2+} ions and produces qualitatively similar conformational changes (see below). More specific information concerning Tb^{3+} and Eu^{3+} binding to TnC was recently reported by Wang et al.[166] based on competitive Ca^{2+} titrations of Tb_x-TnC where x ranges from 2 to 6. By following the Ca^{2+} displacement of Tb^{3+} or Eu^{3+} either by monitoring tyrosine-sensitized metal luminescence or by direct pulsed-laser excitation of the metal, they were able to calculate binding constants for Tb^{3+} and Eu^{3+} to both classes of Ca^{2+}-binding sites on TnC (Table 5). Whether lanthanide ions can substitute for Ca^{2+} in activating actomyosin ATPase activity remains an open question. A

Table 5
BINDING CONSTANTS FOR TERBIUM AND EUROPIUM BINDING TO TnC[166]

Sites	K_{Tb} (M^{-1})	K_{Eu} (M^{-1})
Ca^{2+}-Mg^{2+}	5.2×10^8	4.7×10^9
Ca^{2+}-specific	9.7×10^6	5.3×10^7

THE EFFECT OF Ca^{2+} AND UREA ON COMPLEX FORMATION BETWEEN TnI AND TnC FRAGMENTS

NAME	PEPTIDE STRUCTURE	-UREA +Ca^{2+}	-UREA -Ca^{2+}	+UREA +Ca^{2+}
	I II III IV			
TnC	1–159	+	+	+
TR_2C	89–159	+	+	+
TR_2E	101–159	+	±	-
CB_9	84–135	+	-	-
$TR_2'E$	101–153	±	-	-
TH_2	121–159	-	-	-
TH_1	1–120	+	-	+
TR_1E	1–100	+	-	+
TR_1C	9–84	+	-	+
CB_8	46–77	+	-	-

FIGURE 7. The effects of Ca^{2+} and urea on complex formation between TnI and fragments of TnC. Roman numerals denote binding sites; arabic numerals, residues. Urea, when present, is at a concentration of 8 *M* (see text).[169]

recent paper by Ooikawa et al.[167] suggests that lanthanides inhibit activity; however, the extremely high metal concentrations used in their studies make the physiological relevance of their findings questionable.

9. Regions of the TnC Sequence that Interact with the Other Troponin Subunits

Proteolytic fragments of TnC have provided considerable information on those stretches of its amino acid sequence that constitute sites of interaction with the other troponin subunits, TnI and TnT. TnC can form a binary complex with TnI that is stable both in the absence and presence of Ca^{2+}. In denaturing solvents (e.g., 6 *M* urea), however, Ca^{2+} is required to maintain the stability of the complex.[110] The binding of a variety of TnC fragments to intact TnI under various conditions[109,168,169] has provided evidence that three different regions of TnC are involved: helix II_N, residues 50 to 60; helix III_N, residues 90 to 100; and helix IV_N, residues 126 to 136 (see Figure 7). Of the three regions, only the last appears to bind TnI whether or not Ca^{2+} is present;[169] this would account for the stability of the complex of TnI with native TnC in the absence of the cation. Interactions with TnI involving regions III_N and II_N require the binding of Ca^{2+} to the Ca^{2+}-Mg^{2+} and Ca^{2+}-specific sites, respectively. The former site appears to be responsible for the stability of the TnC-TnI complex in 6 *M* urea (note in Figure

7, for example, that the fragment TR2C, containing region III_N, forms a urea stable complex in the presence of Ca^{2+}, whereas fragment TR2E, in which region III_N is missing, does not); region II_N is likely to function in thin filament activation. The assignment of interaction zones in various segments of the primary structure is consistent with chemical studies. The reactivities towards alkylation of six of the surface lysine residues on TnC are reduced upon formation of a 1:1 complex with TnI. The six are to be found in the three putative interaction sites indicated by the fragment studies.[141]

The two regions in which binding to TnI is Ca^{2+} dependent comprise stretches of the TnC sequence in which structure or accessibility are known to be affected by Ca^{2+} binding. Thus, residues 90 to 100 (III_N), which exist in an unordered configuration in the apo-protein and become folded into an α-helix upon Ca^{2+} or Mg^{2+} binding to sites III and IV,[111] comprise one of the Ca^{2+}-dependent TnI binding sites. In the case of residues 50 to 60, containing the other Ca^{2+}-sensitive TnI binding site, although the α-helical structure is not appreciably affected by Ca^{2+}, tertiary structural changes occur when Ca^{2+} binds to sites I and II,[142] resulting in an increased solvent exposure. This increased solvent exposure deduced from ^{1}H-NMR spectra is also reflected by the increased reactivity of Lys 52.[141] In contrast to the two Ca^{2+}-sensitive TnI binding sites, the Ca^{2+}-independent site is in a region (residues 126 to 136) in which structure and accessibility appear unaffected by Ca^{2+} or Mg^{2+}.[111,132]

Although the three sites differ in their structural dependence upon Ca^{2+}, they do possess similarities that may provide some insight into their TnI-binding properties. In the presence of Ca^{2+}, all have similar distributions of acidic residues aligned along one surface of their respective helices (Figure 8). These residues may form bonds with the abundant basic groups on the TnI surface.

It is more difficult to pinpoint specific sites of interaction between TnC and TnT than between TnC and TnI. Although TnT forms a binary complex with TnC that is stable with or without Ca^{2+}, none of its proteolytic fragments do.[169] In 1978, Leavis et al.[109] found a fragment of TnC comprising residues 121 to 159 that formed a weak complex with TnT, but a more recent study[169] casts doubt on the significance of this interaction. Instead, it now appears that one or more sites exist on the NH_2-terminal half of TnC that form Ca^{2+}-dependent links. For example, TR1C (residues 9 to 84) forms a weak interaction while TR1E (residues 1 to 100) binds more strongly. This observation, along with the finding that Lys 84, 88, and 90 have reduced reactivities in the TnC-TnT complex relative to TnC alone, implicates region 84 to 100 as a second site even though it is already implicated in binding to TnI. The cross-linking studies of Ohara et al.[170] support the view that sites of interaction with TnT exist in both halves of the TnC molecule.

C. Troponin I

A protein that inhibits the Mg^{2+}-stimulated ATPase activity of actomyosin was first isolated by Hartshorne and Mueller[53] and independently by Schaub and Perry;[52] it became known as troponin B or inhibitory factor. This factor was subsequently shown to be heterogeneous,[56,59] consisting of two protein components with molecular weights of 37,000 and 24,000. Greaser and Gergely[60] first demonstrated that the ability to inhibit ATPase activity resided in the 24,000-dalton component which they named troponin I (TnI).

Maximal inhibition by TnI in the absence of the other regulatory proteins of the thin filament requires approximately one TnI per actin monomer; the inhibition is, as might be expected, independent of the presence of calcium.[59,171] If tropomyosin is added to the system, the number of TnI molecules necessary for maximum inhibition is about half that in its absence,[171] indicating that tropomyosin extends the TnI inhibition over several actin monomers in the actin chain. If TnC is now added to such a system, the

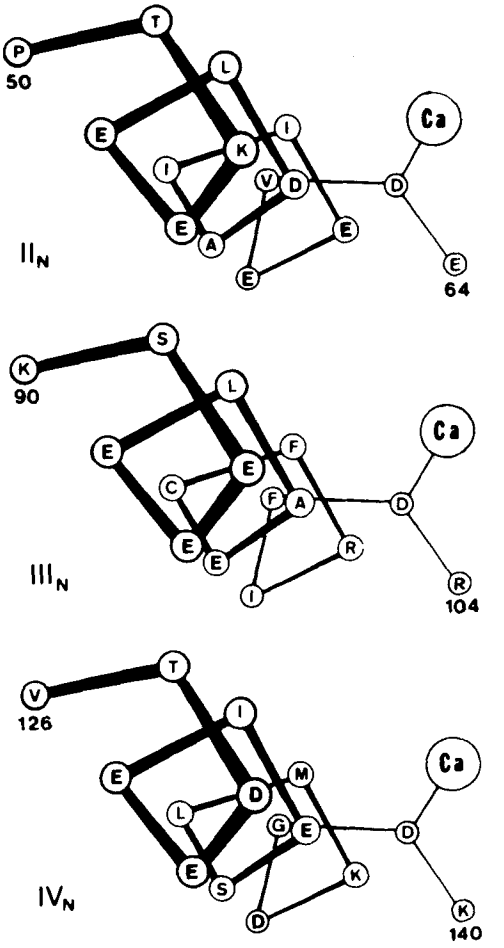

FIGURE 8. Representation of the relative positions of amino acids in alpha-helical segments II_N, III_N, and IV_N. For purposes of comparison, all three helices are shown in the same spatial orientation.[169] Note the alignment of Glu (E) and Asp (D) residues in each helix (drawn from coordinates given in Reference 94).

inhibition is neutralized whether or not Ca^{2+} is present.[168] The TnC-TnI complex is bound to the actin-tropomyosin filament in the absence of Ca^{2+}, but dissociates in its presence.[172] The addition of the third troponin component, TnT, is necessary for the reversible Ca^{2+}-sensitive inhibition.[59] When TnC is replaced with the homologous Ca^{2+}-binding protein calmodulin in the complex with TnI, calmodulin confers Ca^{2+} sensitivity on the ATPase activity.[173] In this case, however, TnI alone was bound to actin in the absence of Ca^{2+} when the system was inhibited. TnI combined with calmodulin and was dissociated from the filament in the presence of Ca^{2+} when the system was active. Thus, TnI could not bind simultaneously to both calmodulin and actin, although it can, in the absence of metal ions, bind to both TnC and actin; the formation of the TnC-TnI complex does not require Ca^{2+}.

TnI is a single polypeptide chain containing 179 amino acid residues (Figure 9). Its molecular weight, calculated from the sequence,[174] is 20,864, somewhat lower than the 21,000 to 24,000 daltons estimated from its mobility on polyacrylamide gels.[53,56,60] The

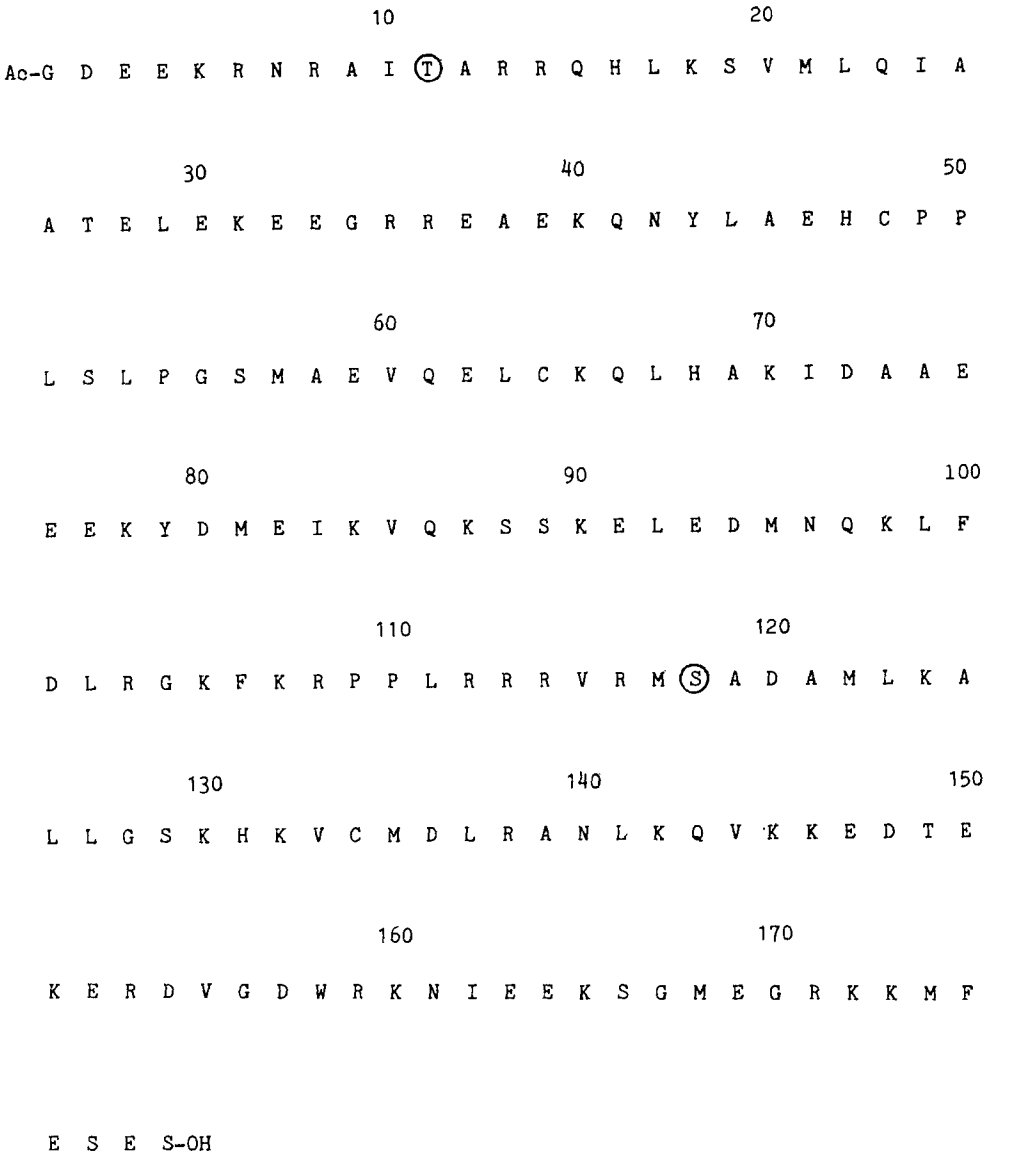

FIGURE 9. Amino acid sequence of rabbit skeletal TnI.[174] Circled residues are phosphorylation sites (see text).

protein has 44 basic and 35 acidic amino acid residues which accounts for its isoelectric point of 9.3.[175] Other features of its amino acid composition include two tyrosyl residues (#43, #79), a single tryptophyl (#158), and three cysteinyl residues (#48, #64, and #133).[174] It has recently come to light that the oxidation state of the cysteinyl residues is related to its ability to form functional complexes with the other troponin subunits.[176,177] Only complexes that incorporate reduced TnI appear to regulate ATP hydrolysis when mixed with actin and tropomyosin.[176] Air oxidation of isolated TnI, which leads to the loss of biological activity, produces structural changes. Thus differences are found in both near and far UV circular dichroic spectra of binary and ternary complexes of TnI with the other subunits depending on whether reduced or oxidized TnI is in the complex.[176,177] Furthermore, the far UV CD spectrum of the reduced

carboxamidomethylated cardiac TnI differs substantially from that of the unmodified, presumably oxidized, protein.

The amino acid sequence in TnI contains two stretches of residues containing unusually high densities of basic residues.[174] The most basic sequence comprises a stretch of 34 residues, from Leu 102 to Met 135, containing 12 basic and only a single acidic side chain. A second region from Lys 5 through 27 contains six basic and no acidic side chains. It is interesting that these two stretches have been identified as sites of interaction with other thin filament proteins[178] and both contain a residue phosphorylatible by enzymes present in muscle (see below).

Isolated skeletal muscle TnI undergoes phosphorylation catalyzed by phosphorylase kinase, principally at Thr 11. Ser 118 is phosphorylated by a 3′,5′-cyclic AMP-dependent protein kinase isolated from bovine cardiac muscle.[179,180] The rate of phosphorylation at both sites is reduced by formation of the TnC complex.[181,182] This must be due to the interaction of the subunits since TnC does not directly affect the activity of the phosphorylating enzymes since the conversion of phosphorylase a into phosphorylase b by phosphorylase kinase, for example, is unaffected by its addition.[179] Addition of Ca^{2+} to the TnC-TnI complex further inhibits the phosphorylation of Thr 11, although formation of the complex using previously phosphorylated TnI is not hindered.[182] This suggests that although the interaction with TnC renders the site of phosphorylation inaccessible to the enzyme, the site itself is not involved directly in complex formation with TnC. The fact that TnI is partially phosphorylated in freshly isolated fast skeletal troponin (~0.5 mol phosphate per mole TnI[182]) leaves open a possible physiological role for the reaction. At any rate, phosphorylation provides a useful probe of the environments of Thr 11 and Ser 118. In cardiac muscle TnI,[183] serine residues at positions 20 and 146 in the amino acid sequence constitute sites for 3′,5′-cyclic AMP-dependent protein kinase catalyzed phosphorylation;[184] Ser 72, Thr 138, Thr 162, and Ser 20 are phosphorylated by phosphorylase b kinase.[185] In the former case, the reaction rate is unaffected by complexation of the TnI with TnC and phosphorylation at Ser 20 appears to decrease the sensitivity of actomyosin ATPase to Ca^{2+}.[186-188]

No direct information on the three-dimensional structure of TnI is available. Early esimates of α-helix and β-sheet content based on Chou and Fasman's[102] predictions from the amino acid sequence[189] or on far UV circular dichroism studies[190,191] are 30 to 50% and 9 to 20%, respectively. One factor possibly contributing to this rather broad range of values, other than the intrinsic uncertainties in the analyses, may be the failure to define the oxidation states of the thiol groups (see above). Four reverse turns in the TnI molecule have also been deduced from the Chou-Fasman analysis:[180] two of these are in regions of the sequence known to interact with TnC and actin, viz., residues 5 to 8 and 109 to 112 (see below).

The selective cleavage of TnI into short peptides using both chemical (cyanogen bromide, cyanylation) and enzymatic (trypsin) reagents has provided a tool for investigating functionally important regions in the sequence. Two peptides, CF2 (residues 1 to 47) and CN4 (96 to 117), have been shown to bind to TnC,[178] indicating that the two proteins may interact at more than one site, a conclusion also arrived at by complementary studies on TnC.[109,169] Studies employing surface labeling of Lys residues of TnI by reductive methylation[192] or alkylation[193] show changes upon TnC binding in the reactivities of Lys residues 31, 40, 98, 105, and 107, all located within the two peptides above. Changes upon complexation in the reactivities to acetic anhydride of Lys 70 and 78 indicate a third region of possible contact between the two proteins.[193]

The CN4 peptide of TnI also binds to actin and retains 40 to 75% of the inhibitory activity of intact TnI on Mg^{2+}-stimulated actomyosin ATPase activity. Also, as is the case with TnI, the inhibitory activity of CN4 is greatly enhanced by the addition of tropomyosin.[178] Thus, this rather remarkable peptide, only 21 residues long, binds to both TnC and actin and inhibits ATP hydrolysis.

FIGURE 10. TnI peptide CN4 (residues 96 to 116). Circled residues (all Arg) are those whose ^{1}H-NMR resonances are perturbed by the addition of actin to the peptide: boxed residues are those perturbed by the addition of TnC. (From Grand, R. J. A., Levine, B. A., and Perry, S. V., *Biochem. J.*, 203, 61, 1982. With permission.)

Recent studies aimed at the further understanding of the mechanism of the inhibitory action of CN4 have involved the synthesis of analogs of the peptide. Talbot and Hodges[194] have produced a 20-residue analog that preserves the exact sequence from Lys 98 through Arg 113, but contains four residues (Arg-Val-Arg-Ala) corresponding to positions 114 to 117 that are not the same as those in the native sequence. This peptide exhibits inhibitory behavior similar to that of Cn4, suggesting that the last four residues are not critical for activity. A peptide corresponding to residues 101 to 115 was shown by Nozaki et al.[195] to inhibit actomyosin ATPase; however, two smaller peptides, residues 101 to 109 and 110 to 115, were inactive when tested separately or in equimolar mixtures. Thus, the minimum "critical" peptide preserving CN4-like behavior appears to be the tridecapeptide, 101 to 113. Recently, on the basis of ^{1}H-NMR studies, Grand et al.[196] have identified specific residues in CN4 that are perturbed by interaction with either TnC or actin (Figure 10).

D. TnT

TnT, the tropomyosin-binding subunit of the troponin complex, is the largest of the three components, consisting of a single polypeptide chain with 259 amino acid residues and a molecular weight of 30,503 (Figure 11).[197] Although the protein possesses an almost equal number of acidic and basic amino acid side chains (61 Asp + Glu, 64 Arg + Lys and 6 His[197]), the isoelectric point, determined by isoelectric focussing, is 9.1.[175] TnT has a high polar amino acid content (~50%) and at physiological pH values the charged residues are distributed more or less evenly throughout the sequence with the exception of the terminal regions: at the NH_2-terminus (residues 1 to 39) acidic residues predominate (18 Glu + Asp, 4 His, no Arg or Lys) and at the COOH-terminus (residues 221 to 259), a stretch of 13 basic, with only two acidic, residues occurs.[197] The even distribution of the remainder of the charged residues suggests that TnT is an open structure with extensive solvent interactions. A Chou and Fasman[102] prediction of the secondary structure yields 37% α-helix and 10% β-sheet with seven β-turns[197] which agrees well with the circular dichroic spectral analysis of TnT by Wu and Yang[191] who obtained 38% α-helix and 14% β-sheet. Although the overall organization of the protein is unknown, a circular dichroism analysis of TnT proteolytic fragments[198] indicates that most of the α-helical structure is localized within residues 71 to 151 and 159 to 227, both sites of TnT binding to tropomyosin (see below).

The hydrodynamic and spectroscopic studies of Prendergast and Potter[199] first suggested that the shape of TnT is elongated and rod-like rather than ellipsoidal. This view of the protein was given additional credence by the recent work of Ohtsuki[200] using antibodies against two chymotryptic TnT fragments, T_1 and T_2, corresponding approximately to the NH_2- and COOH-terminal halves of the protein. Electron micrographs of thin filaments treated with these antibodies showed transverse striations in which positions with respect to the H-zone of the sarcomere depended upon whether anti-T_1 or anti-T_2 was used. Thus, the first anti-T_1 striation occurred 40 nm from the filament end whereas that for anti-T_2 occurred at 27 nm (Figure 12). This result indicates that the TnT molecule indeed extends over an appreciable distance along the thin filament and suggests that the length of the TnT rod is 13 nm or more. More recently, Flicker et al.[200a] have provided direct visualization of troponin. Their electron micro-

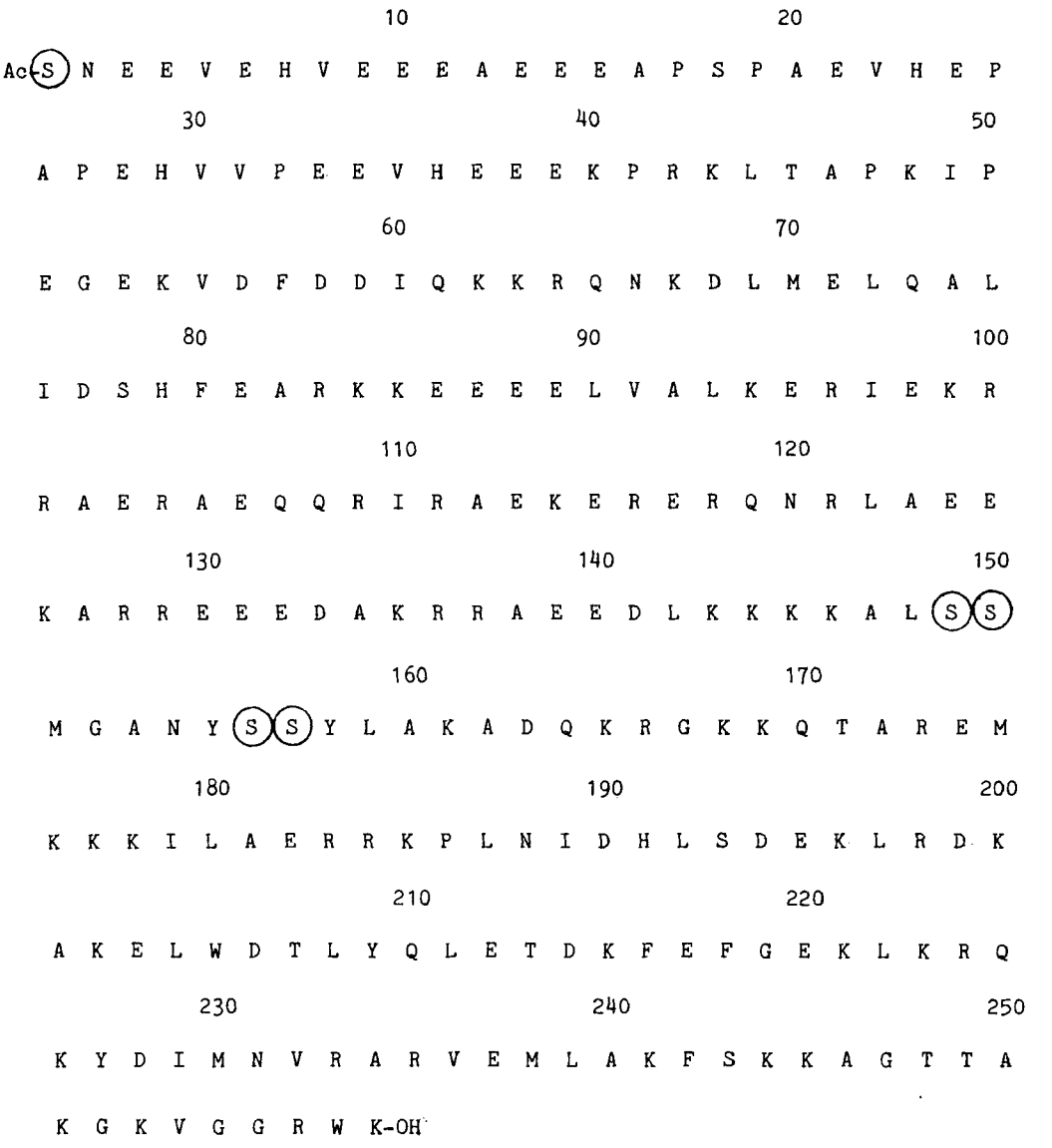

FIGURE 11. The amino acid sequence of TnT.[197] Circled residues are phosphorylatable (see text).

graphs of rotary shadowed molecules reveal the troponin complex to contain a globular moiety and a rod-like tail portion. The globular part of the molecule appears to contain the TnC and TnI subunits while the tail is identified as TnT and is approximately 185 Å long by 20 Å wide.

TnT is present in a phosphorylated form (at Ser 1) in the native troponin complex.[181,201] If isolated TnT is incubated with phosphorylase kinase, not only Ser 1 (phosphorylation site I), but two other groups in the protein, identified as Ser 149 or 150 (phosphorylation site II) and Ser 156 or 157 (phosphorylation site III[197,201,202]), can be phosphorylated. Addition of TnC or TnI to TnT decreases the rate of in vitro phosphorylation by phosphorylase kinase at all three sites.[201] The recent work of Risnik et al.[202] has suggested that while phosphorylation at sites II and III is catalyzed by phosphorylase kinase, that at Ser 1 is catalyzed by a specific TnT kinase present as a contaminant in standard phosphorylase kinase preparations. No significant phospho-

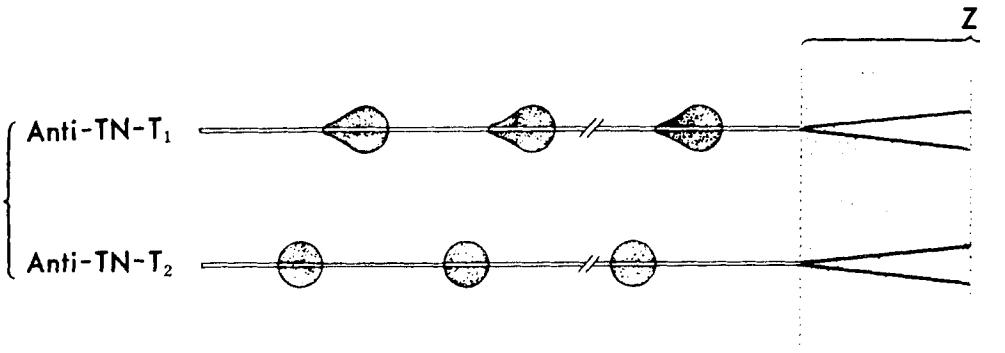

FIGURE 12. Diagram illustrating the distribution along the thin filaments of antibodies raised against the T_1 and T_2 fragments of TnT. The polarity of the filament is indicated by its attachment to the Z-band. (From Ohtsuki, I., *J. Biochem.*, 6, 491, 1979. With permission.)

rylation of TnT is catalyzed by 3′,5′-cyclic AMP-dependent protein kinase.[181] So far no physiological role for TnT phosphorylation has been found.

TnT binds directly to TnC, TnI, and tropomyosin.[52,60,203-205] In the case of TnC, the in vitro formation of a binary complex has been shown in some studies to require Ca^{2+} concentrations in excess of physiological levels in active muscle.[200,203,204] Grabarek et al., however, have demonstrated complex formation even in the absence of metal ions.[169] Cross-linking studies carried out on native troponin do indicate some TnC-TnT contact, although the major cross-linked products are TnC-TnI and TnT-TnI.[206,207] Several proteolytic fragments of TnT bind to TnC in the pesence of Ca^{2+} as demonstrated by affinity chromatography.[198,200] These include the peptic fragment (P2 (residues 159 to 209),[198] a peptide (B2, residues 206 to 258) produced by cleavage at Trp 205 by 2-(2-nitrophenylsulfenyl)-3-methyl-3′-bromoindolenine, i.e., BNPS-skatole[198], and both subfragments of the chymotryptic T_2 peptide of Ohtsuki,[200] which have recently been identified by Pearlstone and Smillie[208] as residues 159 to 227 and 228 to 259. Of the two, the 159 to 227 fragment binds to TnC more strongly.[200] All of the above TnC-binding peptides are derived from the stretch between residues 159 and 259, viz., the carboxy-terminal 2/5 of the TnT molecule. The binding between TnC and TnT is also supported by changes in the reactivities of residues in the latter. Hitchcock et al.[209] have recently shown that the reactivity to alkylating agents of a number of Lys residues in TnT is decreased upon complexation with TnC. Most are spread throughout the region of the sequence comprising the above fragments and include Lys 176, 178, 185, 196, 200, 202, 223, 226, and 241. As mentioned above, phosphorylation at sites II and III is depressed in the TnC-TnT complex, possibly implicating Ser 149 to 150 and 156 to 157 as regions of contact.

The interaction with TnI appears to involve more than one region in TnT. Thus both fragment CB3 (a cyanogen bromide fragment containing residues 1 to 70) and P2 (residues 159 to 209) of TnT interact with TnI as shown by gel chromatography.[210] The first fragment contains the highly acidic stretch of residues (1 to 39) mentioned previously, while the second is the peptic fragment that also binds TnC. A smaller CNBr peptide, CB5 (residues 152 to 175), partially overlaps the sequence of P2 and has been shown[198] to bind to TnI but not to TnC, whereas another peptide, CB4 (residues 176 to 230), binds to TnC but not TnI. Thus it appears that the P2 peptide may bind to TnI at its NH_2-terminal end and to TnC at its COOH-terminus. Again, lysine reactivity studies (decreased reactivites of Lys 54, 62, 63, 176, 178, 185, 196, 200, and 202)[209] as well as the phosphorylation studies[201] on the TnT-TnI complex support the conclusions derived from the fragment work.

Multiple sites of interaction appear to be characteristic for proteins of the thin filament, as further illustrated by TnT binding to tropomyosin. The strong binding of the

TnT fragment CB2 (residues 71 to 151) to tropomyosin points to one region.[211-213] This region of TnT contains a large fraction of the α-helical structure of the protein and Nagano et al.[214] have suggested that its interaction with tropomyosin may involve the formation of a triply coiled coil. The second site is included in the chymotryptic T_2 fragment (159 to 259) of Ohtsuki[200] and has recently been narrowed to the 159 to 227 subfragment.[208]

III. TROPOMYOSIN

Tropomyosin, discovered and isolated in the 1940s by Bailey,[215] had no known role in muscle until the early 1960s. Ebashi and colleagues[10,11,216] first showed that the activity of purified actomyosin from rabbit skeletal muscle showed a dependence on Ca^{2+} only when another component that they called native tropomyosin was present in the system. Native tropomyosin was subsequently shown to consist of Bailey's tropomyosin plus another protein factor named troponin by Ebashi and Kodama.[11] A number of early papers, notably those of Martonosi[217] and Laki et al.,[218] indicated that tropomyosin was associated with the actin filaments. Hanson and Lowy[16] were able to visualize the arrangement of actin monomers in micrographs of negatively stained actin filaments and described their structure in terms of a two-stranded helix. Tropomyosin may occupy the long pitched groove between the coiled strands. This picture was further refined by three-dimensional image reconstructions from electron micrographs[219] and by X-ray diffraction studies[220,221] leading to the conclusion that the tropomyosin molecules actually lie slightly out of the groove in closer association with one actin chain than with the other. The implications of this view for regulatory mechanisms will be discussed below.

A. Chemical Properties and Structure

Tropomyosin is a fibrous molecule of about 66,000 daltons. In denaturing media, under reducing conditions, it dissociates into two 33,000 dalton subunits.[222] Subsequent work has shown that two types of subunits exist, designated α and β,[223] which form either homo $\alpha\alpha$- and $\beta\beta$- or hetero $\alpha\beta$-dimers.[224,245] A comparison of the amino acid sequences of the α and β components reveals a high degree of similarity. Of the 284 residues,[225-227] there are 39 substitutions, essentially all conservative (i.e., chemically similar side chains, see Figure 13). The substitutions of Glu for Ala 41 and Ser 229 and Asn for His 276 in the β subunit results in a change in the net charge that, presumably, makes it possible to separate the two chains by ion exchange chromatography in the presence of urea.[223] Another difference between the two chains is the presence of a Cys residue at position 36 in the β chain in addition to Cys 190, which is common to both chains.

The significance, if any, of the existence of two different tropomyosin chains is not clear, although slight differences have been reported in their physicochemical properties. The ratios of the α and β forms present in a particular muscle appear to depend upon its type.[228-230] In fast twitch skeletal muscle, $\alpha/\beta \sim 4$ and the predominant molecular species is $\alpha\alpha$, with some $\alpha\beta$ present.[224,245] Although in both slow twitch striated and smooth muscles the ratio is closer to 1,[223,228] some workers have suggested[230-232] that α-tropomyosin is confined to type II and β-tropomyosin to type I fibers, implying that the two cell types contain $\alpha\alpha$, and $\beta\beta$-tropomyosin, respectively. This finding has, however, been contradicted by analyses of the proteins in typed single fibers showing essentially equal amounts of the α and β forms in both fiber types.[233] Finally, it has been shown that embryonic muscle cells contain a high proportion of β chains and the α/β ratio changes during development.[231,234] Furthermore both α and β subunits may

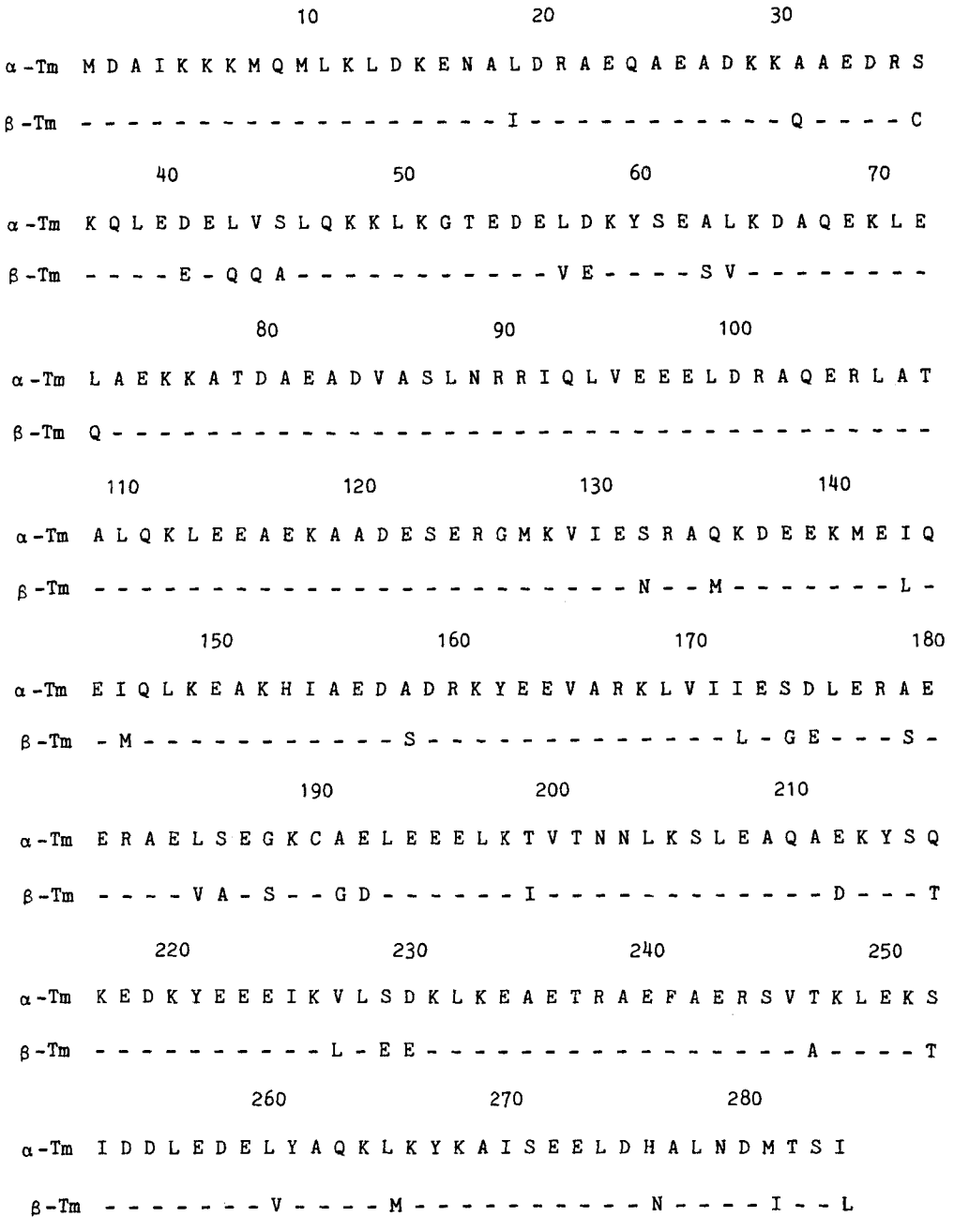

FIGURE 13. Amino acid sequences of the α-, and β-chains of rabbit skeletal tropomyosin.[227] Only those residues are shown in the β-tropomyosin sequence that differ from their counterparts in the α-chain.

exist in two variants, a phosphorylated and a nonphosphorylated form, in various developing muscle types.[235]

Both chains have a high α-helix content (>90%);[236] they are arranged in the intact tropomyosin molecule as a two-stranded coiled-coil[225,237] with its structure stabilized primarily by hydrophobic interactions between regularly spaced nonpolar side chains in each of the two strands[225,226] and secondarily, by coulombic interactions between vicinal charged side chains.[238-241]

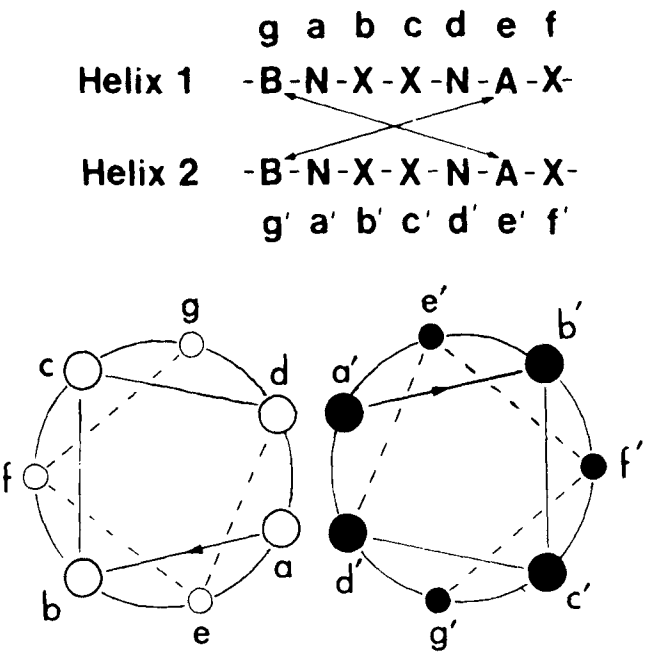

FIGURE 14. Top: The heptapeptide repeat in tropomyosin. The seven residues are designated a to g with nonpolar residues (N) found at positions a and d and a high proportion of basic (B) and acidic (A) residues at positions g and e, respectively. The possible ionic interactions that can occur between the g and e positions of the two helices of the coiled coil are indicated by the double-headed arrows. Bottom: Axial view of the two coiled coil helices looking from the NH_2-terminal end. The coiled coil structure is stabilized by interactions of nonpolar core residues in positions a and a′ and d and d′. Electrostatic interactions occur between g and e′ and e′ and g′.[239,240] (From Smillie, L. B., *Trends Biochem. Sci.*, 4, 151, 1979. With permission.)

The amino acid sequence of tropomyosin has provided insight into the details of interchain interactions.[225,226] Throughout the entire 284-residue sequence of each chain, a regular pattern of repeating heptapeptides emerges in which nonpolar residues are found at positions a and d[241] (Figure 14). Furthermore, a high proportion of basic amino acids occurs in position g and acidic ones in position e. Since the two helical chains run in the same direction with 3.6 residues per turn, nonpolar residues from one chain face non-polar residues from the other in the surface of contact between the two. The basic residues in position g of one chain line up with acidic residues in position e of the preceding heptapeptide in the other chain leading to stabilizing electrostatic interactions.[241] The overall structure corresponds to the classic "knobs and hole" type of alignment proposed in 1953 by Francis Crick[242] for coiled-coil structures in general.

Because of the quasi-equivalent repeating heptamers, the interacting amino acid residues in the two tropomyosin chains could, in principle, be brought into proximity when the two chains are aligned either in register or staggered with respect to one another by any integral multiple of seven residues. An early analysis of the sequences yielded a model in which a 14-residue stagger[226] was most favorable in that it optimized hydrophobic contacts between the chains. This model was also considered attractive because it contained single-stranded stretches of residues at either end of the molecule, allowing for the head-to-tail aggregation. However, overwhelming evidence from subsequent studies for the chains being in register included the observations that: (1) complementary Cys 190 residues in the two chains can be cross-linked either by air[243,244] or disulfide exchange[245] oxidation, (2) the near ultraviolet circular dichroism and absorption spectra[246,246a] of tropomyosin tyrosyl residues indicate ring-ring interaction in

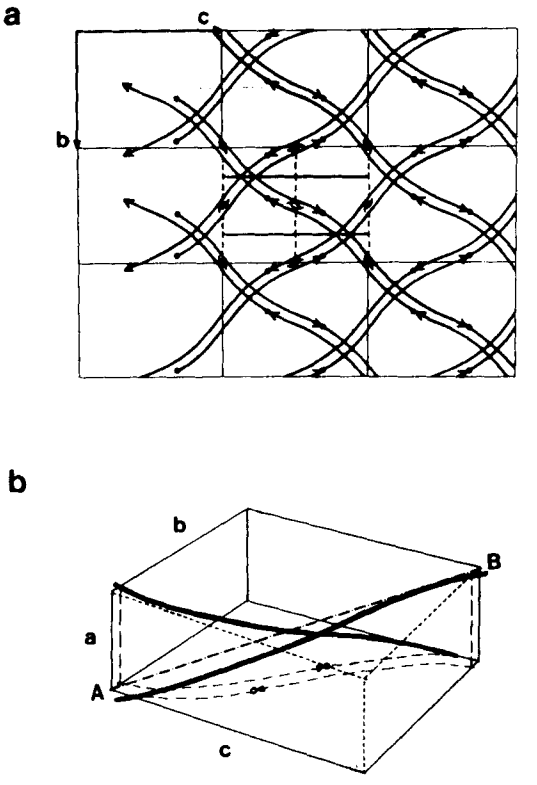

FIGURE 15. (a) The unit cell and locations of tropomyosin molecules in the kite-shaped crystal lattice. The arrowheads indicate the orienation but not the ends of the molecules in the filaments. (b) The unit cell in (a) drawn in three dimensions to show the direction of the tropomyosin strands (line AB) along the body diagonal of the cell. The dashed lines in the bc plane show the projection of the tropomyosin strands as seen in (a). Only two of the several tropomyosin strands in the unit cell are shown for clarity.[255,443] (With permission from Caspar, D. L. D., Cohen, C., and Longley, W., Tropomyosin: crystal structure, polymorphism and molecular interactions, *J. Mol. Biol.*, 41, 87, 1969. Copyright: Academic Press Inc. (London) Ltd.)

complementary strands, and (3) the addition of pyrene maleimide labels to the two Cys 190 residues results in the appearance of an exited state dimer.[247,248] In-register alignment is also favored because the staggered model is too long to fit the crystal and paracrystal repeat distances.[241] Furthermore, the nonstaggered model would provide pseudoequivalent surfaces for each of seven actins and matches the troponin repeat distance.[249]

Considerable information concerning the physical properties of the tropomyosin molecules has come from the studies on tropomyosin crystals from a variety of species.[250-255] These crystals, which contain 95% water, are made up of a network of filaments formed by molecules bonded head-to-tail, running along the body diagonal of the unit cell. Cross-connections between tropomyosin strands give rise to a kite-shaped mesh in electron micrographs. Each strand in the electron micrograph (EM) represents two filaments running along opposite body diagonals. The distinctive kite shape of the mesh, which is due to a sinusoidal bend in the filaments, gives rise to short and long arms in the EM pattern (Figure 15). With the use of mercury as a marker of the locations of the cysteine residues,[253,255] Cys 36 has been located within the short arm, whereas Cys 190 occurs at the approximate midpoint of the long arm. This information plus that obtained by matching the predicted and actually obtained electron density maps along the filament axis have led to the conclusion that the molecule begins

and ends in a short arm. Two strands cross the molecule, one about one third of its length from the NH_2-terminus, the other close to the COOH-terminus.[255]

A single molecule within the filament is 410 ± 4 Å long.[250] Since a fully helical 284-residue molecule would be 423 Å long, there appears to be an eight- to nine-residue overlap at the ends of the molecule. This implies that in the thin filament, the tropomyosin axial repeat of 385 Å results from twisting of the supercoil when it is bound to the actin helix. Phillips et al.[253] have calculated an average radius of bending of 45.2 Å for the actin-bound tropomyosin, about four times the radius of the sinuosoidal bend in the crystal lattice. Thus, the molecule must contain regions in its structure with a high degree of flexibility, a supposition supported by observed slight irregularities in electron density maps,[253] by diffuse scattering in X-ray photographs suggesting molecular vibrations,[255] and by changes in bending of the tropomyosin induced by the addition of troponin to the crystal.[251,252,256]

Further evidence for the flexibility comes from fluorescence anisotropy decay studies on labeled tropomyosin in solution. Wahl et al.[257] showed that both the monomer and polymer (head-to-tail) have rotational correlation times considerably less than those expected for rigid molecules of the same size, indicating axial bending along the entire length of the molecule. Troponin addition decreased this axial bending of tropomyosin and this effect was partially reversed by adding calcium to the system.

One site of local instability in tropomyosin appears to involve the region containing Cys 190. Several studies using fluorescent[248,258] or spin labels[259,259a] specifically attached to the Cys 190 residues within a tropomyosin molecule have shown that guanidinium chloride or thermal denaturation results in a local change in the immediate environment of the probe that precedes the more generalized melting of the structure and dissociation of the two chains. This local change typically occurred at ~1.0 *M* guanidinium chloride or at temperatures between 30 to 35°C, whereas the global unfolding required >3.5 *M* guanidinium chloride or temperatures >60°C. Further studies employing intrinsic (Tyr) fluorescence polarization,[260] optical rotation,[261-263] and ^{1}H-nuclear magnetic resonance[264] suggest that this loss of structure initiated at Cys 190 is propagated along the length of the molecule towards both the NH_2– and COOH–terminal ends.

Comparison of the denaturation of tropomyosin, in which two chains had been cross-linked by air- or disulfide-exchange oxidation of the two Cys 190 residues with that of preparations in which the Cys residues were kept reduced by the presence of dithiolthreitol or were alkylated,[265] has shed further light on the states of the tropomyosin molecule. Cross-linking shifted the concentrations of guanidinium chloride or the temperature required for the main helix-coil transition to higher values, indicating that the disulfide bond afforded some protection against complete unfolding, but made the intermediate local chain separation around the cross-linked residues more pronounced (Figure 16). This somewhat unexpected result can be explained if one considers that bringing the two thiol groups close enough to interact introduces a strained conformation in the molecule. Space filling models show that in the native coiled-coil α-helical structure, the two groups point away from one another.[266] Further work using pyrene maleimide[247,248] or didansylcysteine[266] to label both Cys 190 residues suggests that the (labeled) protein can exist in two states: in one the molecule is fully helical and the thiols are further apart; in the other local chain separation occurs that allows the probes to interact. In the case of the pyrene label, this interaction gives rise to an excited-state pyrene dimer detectable by its excimer fluorescence,[247,248] while with the dansylcysteine probes, to species with new excited-state lifetimes.[266] The two states exist in equilibrium; increasing temperature and ionic strength shift the equilibrium toward the locally unfolded state. The fact that head-to-tail polymerization of tropomyosin labeled at Cys 190 was inhibited, the degree of inhibition, depending upon the

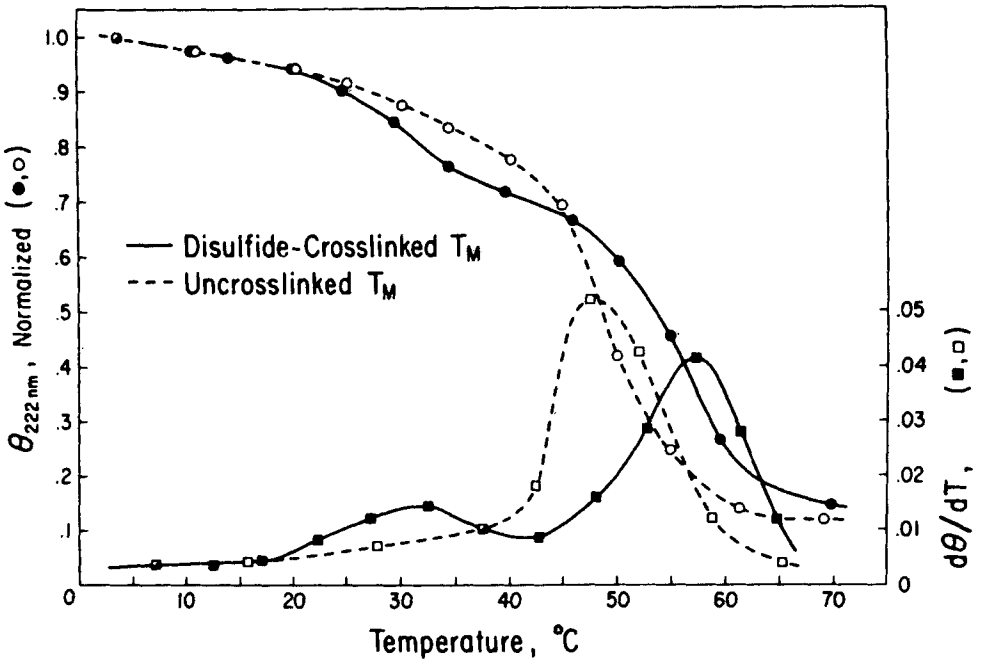

FIGURE 16. Unfolding of uncrosslinked (----) and disulfide crosslinked (——) rabbit skeletal tropomyosin as revealed by circular dichroism. (From Lehrer, S. S., Graceffa, P., and Betteridge, D., *Ann. N. Y. Acad. Sci.*, 366, 285, 1981. With permission.)

label used, implies that local perturbations in the Cys 190 region may exert effects 94 residues away at the COOH-terminus. Similar perturbations may be caused by the binding in this region of troponin. This would be of significance insofar as any factor that influences the head-to-tail polymerization of tropomyosin may also be expected to affect its ability to bind to actin. This will be further discussed below.

As previously mentioned, the length of the tropomyosin molecule in crystals, determined by X-ray studies, is 410 Å compared to a predicted value of 423 Å for a 284-residue helix. Since the chains are in register, this implies that an approximately 13-Å stretch, or 8 to 9 amino acid residues, are involved in the head-to-tail overlap of the molecules. McLachlan and Stewart[241] indeed showed that there was an excellent fit between the broad faces of the two supercoils in which there were several nonpolar and several possible electrostatic interactions, viz., Ala 277 and Ile 284 fit against Mets 1 and 8, respectively; Thr 282 with Met 10; and Asp 280, His 276, and the COOH group of Ile 284 with Lys 6, Asp 2, and Lys 7 (Figure 17). Experimental results consistent with this model were provided by Johnson and Smillie[267] who sequentially removed COOH-terminal amino acids with carboxypeptidase A. Loss of the last four residues, but not the last three, resulted in a loss of polymerizability. This points to Met 281 as also playing a key role in stabilizing the hydrophobic core of the overlap region. The same work also showed that polymerizability was lost when Lys 7 was acetylated, presumably disrupting salt-bridge formation with the COOH group of Ile 284, and when Met 8 was modified. More recently, Mak and Smillie[268] and Trueblood et al.[269] have studied thin-filament interactions using carboxypeptidase A-digested tropomyosin preparations (see below). It should be pointed out here that the recent X-ray work of Phillips et al.[255] does not exhibit the requisite densities at the overlap regions that might be expected from the simple overlap of coiled-coils suggested from the work discussed above.

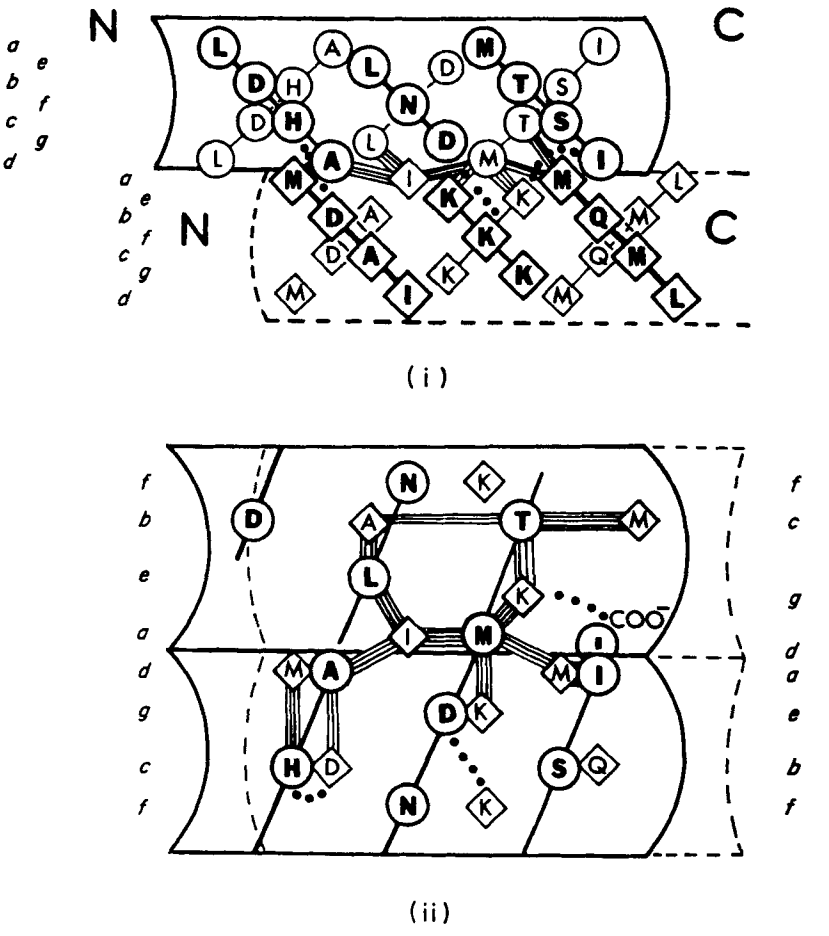

FIGURE 17. Schematic diagram of the end-to-end overlap model of tropomyosin interactions. The broad supercoil face at the carboxyl ends of two parallel unstaggered helices in the left-hand molecule. (◊) overlaps the broad face of the amino end of the right-hand molecule (○). (i) Side view of the narrow edge of the supercoils. Amino acids of the second helix in each molecule are visible behind, drawn with 3.5 residues per turn. (ii) View from above, looking at the broad face. The right-hand molecule is now below. Dotted lines indicate the salt bridges and the shading shows important van der Waals contacts. The region of overlap involves 9 amino acids in each chain and is 13.5 Å long. (With permission from McLachlan, A. D. and Stewart, M., Tropomyosin coiled-coil interactions: evidence for an unstaggered structure, *J. Mol. Biol.*, 98, 293, 1975. Copyright: Academic Press Inc. (London) Ltd.)

B. Sites of Tropomyosin Interaction with TnT and Actin

One troponin complex binds via the TnT subunit to each tropomyosin molecule, examination of crystals indicating that it occupies a position about 100 to 150 Å from the COOH-terminal end of the molecule.[251] Recent studies on tryptic fragments of tropomyosin[213,270,271] further pinpoint the interaction as principally involving, although not exclusively confined to, residues 190 to 284. An intriguing result in these studies is the observation that TnT or CB1 (a TnT fragment comprising residues 1 to 151) can cross-link the NH_2- and COOH-terminal halves of tropomyosin even though neither TnT nor CB1 binds to the NH_2-terminal fragment alone. This finding coupled with the fact that TnT is a rod-shaped molecule at least 130 Å long (see section on TnT) has led to the suggestion that TnT may extend over the entire COOH-terminal one third of the tropomyosin molecule including the region of head-to-tail overlap, thereby stabilizing tropomyosin polymers (Figure 18).[200a]

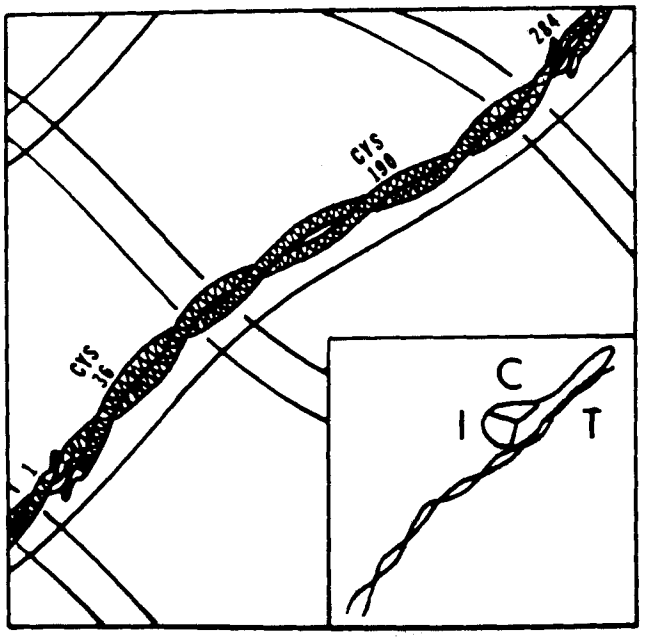

FIGURE 18. A tropomyosin molecule in the crystal lattice showing the positions of the Cys residues (#36 and 190). Inset: The arrangement of the troponin components (I, T, and C) on the tropomyosin filament.[253] (Reproduced from Phillips, G. N., Jr., Fillers, J. P., and Cohen, C., *Biophysical Journal*, 1980, 32, 485, by copyright permission of the Biophysical Society.)

The specific nature of the TnT-tropomyosin interaction remains unknown, although Nagano and colleagues[214] and Mak and Smillie[213] suggest that the tropomyosin-binding region of the TnT is α-helical and may form a triply stranded coiled-coil with the COOH-terminal one third of tropomyosin. This might be expected to affect the conformational dynamics of the molecule resulting in the decrease in flexibility mentioned above.

The fact that tropomyosin makes contact with seven monomeric actin units suggests that some type of equivalent or quasi-equivalent interaction occurs at each of the seven regions of contact. Accordingly, one might expect to see some repeating pattern of surface residues at a repeat distance of approximately 58 Å or one seventh the molecular length of 410 Å. Assuming 1.49 Å as the average residue translation along the coiled-coil axis,[272] the 58-Å repeat would correspond to a periodicity of about 39 residues. Parry[249] and Stone et al.[239] found groupings of nonpolar, acidic, and basic residues with a repeat of 19½ residues extending through the entire sequence, suggesting not 7 but 14 quasi-equivalent spatial arrangements of surface residues. The existence of this pattern is reinforced by Fourier analyses of the distributions of acidic, basic, and nonpolar residues along the chain axis.[273] This revealed a strong 19.73-residue periodicity of acidic residues grouped into 10- to 12-residue wide bands and occurring exactly 14 times in the 410 Å length of each chain (Figure 19). The two chains together in the supercoil would therefore contain 14 pairs of acidic zones, each pair rotated 90° with respect to its neighbors. Stewart and McLachlan[273] speculate that the 14 regions correspond to two sets of actin-binding sites and that these acidic regions along the tropomyosin axis may link to similar acidic sites on actin subunits, the bonds involving Mg^{2+} bridges between negatively charged residues on the two proteins as well as salt bridges and hydrophobic interactions. It has been further speculated that a change

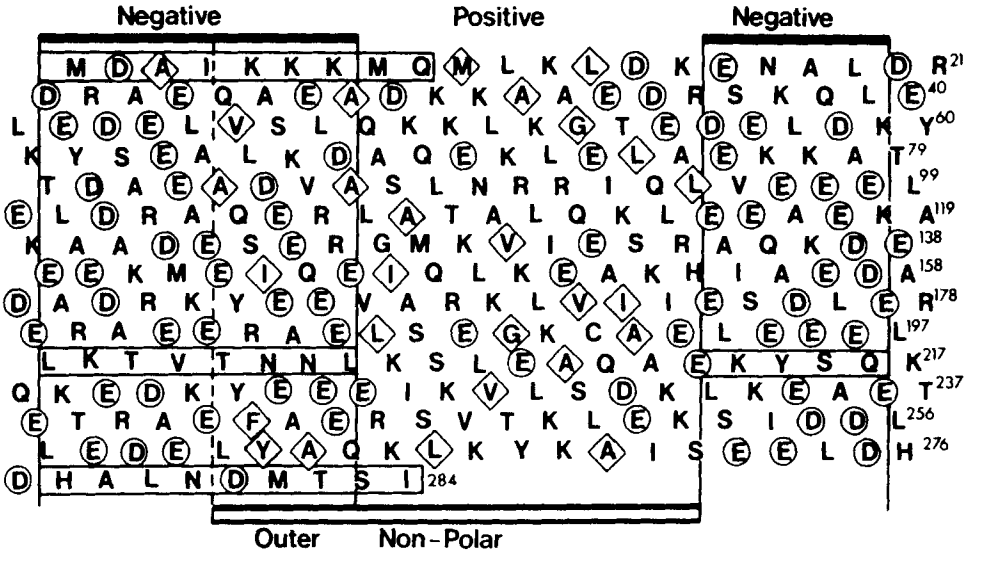

FIGURE 19. Amino acid squence of tropomyosin drawn in the 19⅔ repeat pattern with 14 bands in the first 275 residues. Negative charges are ringed (O) and are concentrated in two zones in each repeat. Nonpolar groups in outer positions b, c, and f of the coiled-coil (see Figure 14) are marked (◇) and lie in a sharply defined hydrophobic zone. Boxes show (i) residues 1 to 9 and 276 to 284, which are believed to overlap in the head-to-tail linkage and (ii) residues 197 to 217 postulated to form the troponin binding site. For clarity, one or more residues that lie near the end of a repeat are also shown at the beginning of the next repeat.[36,444]

from the off-state to the on-state in muscle would involve a one-quarter turn of the tropomyosin to the alternative set of sites.

It should be pointed out that those regions of the tropomyosin molecule with greater internal flexibility (i.e., decreased α-helical stability) in the COOH-terminal third (see above) might contain actin-binding zones that vary in their affinity for actin. Furthermore, any structure-stabilizing effects of troponin binding to this region might modulate the tropomoyosin-actin interaction.[213,251]

IV. ACTIN

The discovery of actin, which is now recognized as the major thin filament protein, by Straub[2] in 1942 in Szent-Gyorgyi's laboratory, opened up a new epoch in the biochemistry of muscle. What in the early literature was described as myosin turned out to be the complex of two proteins, myosin and actin. Actin itself can be extracted from acetone-dried muscle powder with distilled water. It is present in the watery extract as a globular protein, G-actin. The molecular weight of skeletal G-actin, on the basis of its amino acid sequence (Figure 20), is 41,785.[274]

On addition of various salts, conventionally about 0.1 *M* for monovalent cations and about 1 m*M* for divalent cations, a G-actin solution undergoes a change marked by an increased viscosity and the appearance of birefringence of flow, suggesting the presence of large asymmetric molecules. These are so-called F- (for fibrous) actin produced through polymerization of the globular units.[2] The average molecular weight of F-actin is of the order of 3×10^6 (see Reference 275) and its length 10 μm or longer, in contrast to actin filaments in vivo which have a length of 1 μm per half sarcomere.[276] The factors that limit the length of actin filaments in vivo have not been identified, although the involvement of other proteins associated with the filament, e.g., tropomyosin, and α-actinin, has been suggested.[277,278]

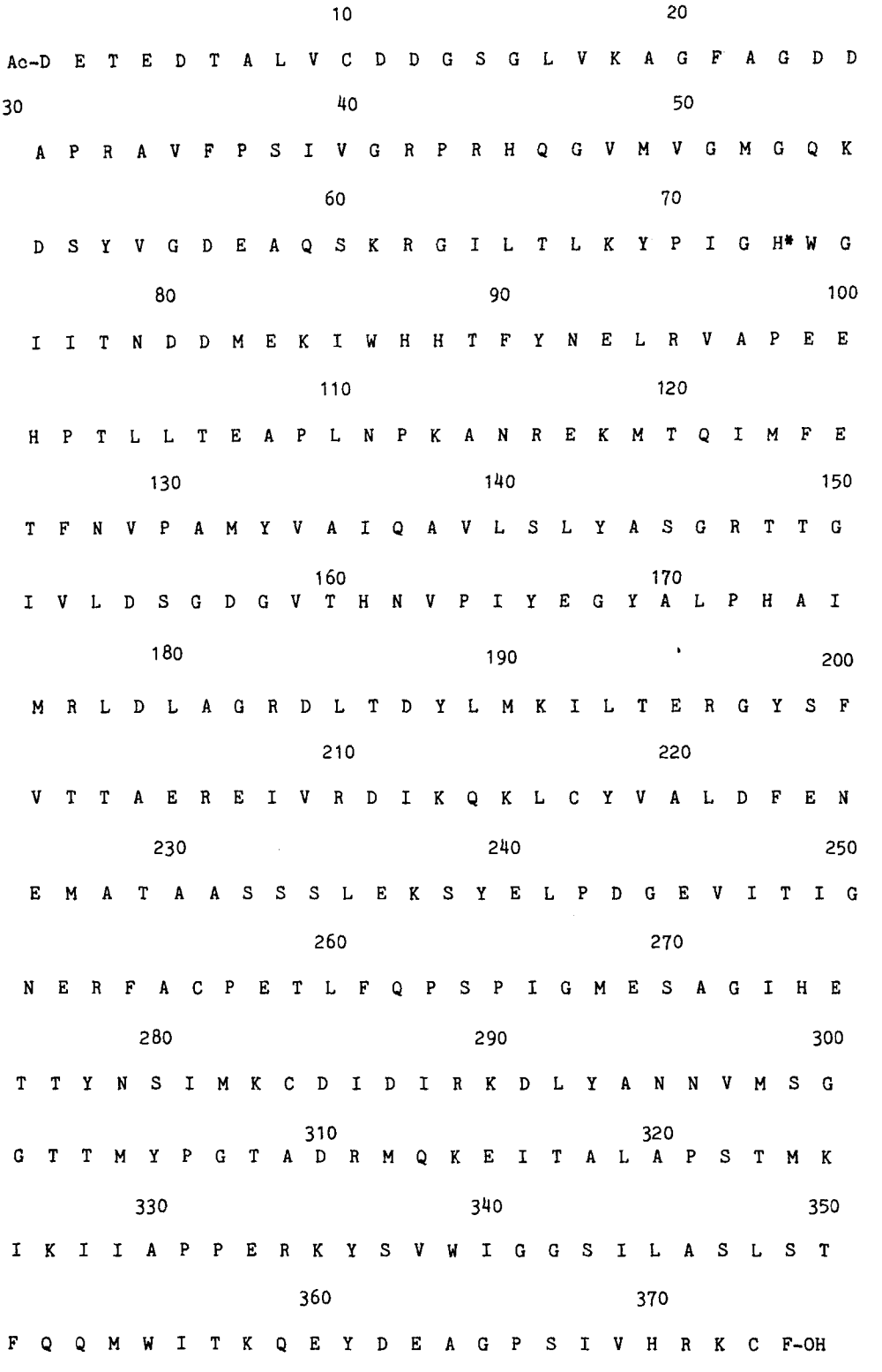

FIGURE 20. Amino acid sequence of rabbit skeletal muscle actin.[274] H* is 3-methyl-histidine.

G-actin contains tightly bound Ca^{2+} or Mg^{2+} ($K_{ass} \sim 10^5\ M^{-1}$).[279-281] and ATP ($K_{ass} \sim 10^{10}\ M^{-1}$),[282,283] 1 mol of each per mole actin, which can undergo rapid exchange with added Ca^{2+} or ATP, respectively,[284,285] as shown by radioactive tracer experiments. Although various studies have suggested that the cation and nucleotide were bound as complex to a single site on the actin molecule,[286-288] recent studies indicate that they bind to different sites on the protein;[289] thus according to a recent NMR study, the two are separated by at least 16 A.[290] Both the Ca^{2+} and ATP bound to actin can be exchanged by other metals and nucleotides, respectively.[291-294] It appears likely that both the bound metal and nucleotide are necessary in G-actin to stabilize its tertiary structure — removal of either leads to the irreversible denaturation of the protein and the loss of its polymerizability.[281,295,296]

Although G-actin was originally thought of as a spherical protein, three-dimensional reconstructions of electron micrographs of actin filaments combined with myosin subfragment-1 (decorated filaments) suggested over a decade ago[219] that actin monomers are somewhat asymmetric with dimensions of 5.5 (axial) × 3.5 (radial) × 5.0 (tangential) nm with respect to the filament axis. More details of the actin structure emerged from electron microscopic studies of gadolinium (Gd)-actin sheets[296a,296b,296c,419] and from X-ray diffraction studies of crystals of the actin-DNase complex.[418] Both approaches led to an actin molecule containing two globular domains separated by a cleft, but the dimensions deduced by the two groups are slightly different; viz., 6.7 × 4.0 × 3.7 nm from the X-ray studies and 5.6 × 4.3 × 3.3 nm from the electron microscopic work.

A more recent reconstruction of the actin monomer, while reaffirming the essential correctness of the earlier description, shows clearly that actin is kidney-shaped, about 6.7 nm in its longest dimension and is flattened to a thickness of about 3 nm.[296d] Each of the two lobes is an elongated domain with its long axes perpendicular to the kidney long axis. The two lobes are of unequal size, the larger of the two having a long axis of about 5.5 nm. The best fit of the monomer structure derived from X-ray crystallography into the F-actin helical structure determined from negatively stained paracrystals[296e] was obtained by aligning the monomer with its kidney axis approximately at right angles to the helix axis. Intermonomer contacts are made between the smaller domains of neighboring subunits and are mostly within 1 nm of the filament axis. The maximum diameter is 10 nm, and the minimum is 6.5 m. The larger domains lie at large radius. The proposed structure allows flexibility in the azimuthal direction, as earlier suggested by Egelman et al.,[415] as well as being consistent with hinges at right angles to the helix axis that would allow deformation of rafts of cross-linked actin filaments from a rectangular to the trapezoidal array.[296f,296g]

The 10-nm filament diameter of this analysis, as well as those of Egelman et al.[296b] involving a simpler two-sphere monomer, and of Egelman and Padron,[296j] based on X-ray diffraction of actin filaments, is significantly larger than the 7- to 8-nm value derived from electron microscopy[15,219,368] and more recently deduced from electron micrographs of Pt-shadowed freeze-dried actin filaments.[296k] If one accepts the smaller diameter, a parallel alignment of the monomer major axis with the filament axis, as previously proposed,[219,296b,296c] would follow.

The F-actin filament, once formed, exhibits considerable flexibility — bending motions were first demonstrated by the quasi-elastic laser light scattering experiments of Fujime and colleagues[296m,296n,379] and later by electron microscopy,[296p] fluorescence polarization of labeled filaments,[296q] saturation transfer EPR of spin-labeled filaments,[404] and most recently by direct microscopic visualization of filaments labeled with a fluorescent probe.[296r] The correlation times characterizing these motions range from seconds to nanoseconds; the motions may involve whole filaments, subunits within filaments, or segments within subunits. In the case of labels, particularly for faster mo-

tions, it becomes difficult to distinguish between motions attributable to the protein and those of the probe itself relative to its attachment site. Addition of tropomyosin and troponin in the absence of Ca^{2+} results in a more rigid filament; however, the addition of Ca^{2+} restores the flexibility to the level of the pure actin filament.[296n,379] In addition to the bending motions along the filament, fluctuations also appear to occur in the twist of the actin helix, i.e., how individual subunits are positioned azimuthally with respect to one another. Although values have been reported for actin filaments in paracyrstals[296r] or in cross-linked bundles[296r] and fibers,[296u] Egelman et al.[415] have recently emphasized the existence of variations in the twist along the axes of isolated filaments, giving rise to fluctuations of about 10° in the azimuthal angle between adjacent monomer units. In addition to the bending and twisting motions of the overall filament, Yanagida and Oosawa[296v] also suggest some degree of rotational freedom of the monomer subunits within the filament on the basis of their fluorescence polarization studies.

A. Actin Polymerization

The polymerization of actin is accompanied by the hydrolysis of the bound ATP.[297,298] ADP remains tightly bound while the phosphate is released. Polymerization stops when the actin monomer concentration drops below the so-called critical concentration, as first shown by Oosawa and colleagues.[299-302] ATP hydrolysis continues after polymerization has stopped,[303,304] the rate of this steady-state hydrolysis, which involves the exchange of G-ADP units in the filament for G-ATP units in solution, depending upon the ionic conditions.[305-307] It has now been established that actin monomers attach and detach at the ends of the filaments.[303,308] which exhibit polarity as shown by the formation of arrowheads on addition of myosin subfragment 1 or HMM. There is good evidence that the monomers attach preferentially to the barbed end of the arrowhead, while the detachment is favored at the pointed end.[309-311]

The first step in the polymerization process is the formation of nuclei consisting of three to four monomers. This process may be accomplished not only by ATP hydrolysis, but P_i may also remain bound at this stage.[312] The existence of G-ATP units in the nuclei has recently been considered.[313,314]

Several recent studies have addressed the possibility of G-actin undergoing changes before nuclear formation. Rich and Estes,[315] in studying the susceptibility of actin to enzymatic proteolysis, discovered a new monomer form that was generated by the addition of KCl to subcritical concentrations of G-actin. The new form, although clearly monomeric and containing bound ATP, showed a decreased susceptibility to digestion, which is characteristic of F-actin. They concluded that this novel actin represented a conformationally distinct form of monomer that precedes nucleation. This view is supported by recent spectroscopic studies;[316] however, it is contradicted by pulse-fluorometry studies on an actin-bound probe that indicated that no change occurred in the G-actin structure until after its incorporation into the filament.[317] If such a prenucleation actin exists, a scheme of polymerization might be[316]

$$\text{G-A} \underset{\text{cations}}{\rightleftharpoons} \text{G*-A} \rightleftharpoons \text{N-A} \underset{\text{G*-A}}{\rightleftharpoons} \text{F-A}$$

monomer monomer nucleus filament

where G*-A is the "activated" prenucleation form.

While end-to-end association of actin filaments has been reported,[303] spontaneous fragmentation of filaments — in contrast to fragmentation produced by mechanical

effects and by ultrasound[318] — has recently been invoked[319,320] to explain certain autocatalytic features of the polymerization process. We shall briefly refer below to various proteins that induce filament shortening.

While polymerization of actin can occur in the presence of nonhydrolyzable nucleotides (e.g., ADP, AMP·PNP),[321,322] the continuous addition of monomers at one end and the removal at the other requires steady hydrolysis of ATP (treadmilling).[303,304,304a] Earlier studies left open whether additon of monomers in the steady state could take place along the length of the filaments (exchange diffusion) in addition to the ends. Wegner,[283] based on the analysis of phosphate liberation and ADP exchange during polymerization, concludes that nucleotide release and rebinding were mainly associated with the filament ends referred to above, pointed and barbed, respectively. The recent work of Brenner and Korn,[322a] however, suggests that exchange diffusion may play a more important role than treadmilling depending on the ionic conditions. Clearly, more work is needed to answer this question.

While the earlier studies by Oosawa's group[299-302] defined the critical actin concentration as that below which no polymerization could take place, Wegner's work[303] and that of Kirschner[323] point to two additional critical concentrations: one — the lowest — at which polymerization at the barbed end begins and another — the highest — at which growth at the pointed end begins. The previously defined net critical concentration — constant length of filaments — falls between the two. Very recently it has been shown that millimolar Ca^{2+} or Mg^{2+} is required for the fast exchange of monomers and that, depending on the divalent metal concentration, the differences in the addition and dissociation rates at the two filament ends can be varied.[306] Since the driving force for the monomer flux through the filament either during elongation or in steady state treadmilling is the hydrolysis of ATP at the elongating end of the molecule, the monomer flux becomes zero as the ATP concentration reaches its equilibrium value.[303,304] Further experiments aimed at a better understanding of actin polymerization, particularly in nonmuscle systems, are necessary and the recent use by Taylor et al.[324] of fluorescence energy transfer between actin monomer units within a filament appears promising.

A variety of factors indigenous to muscle tissue have some regulatory activity in actin polymerization. Thus, in addition to the above-mentioned mono- and divalent cation-induced actin polymerization, several other muscle proteins including myosin,[325] tropomyosin,[326] and α-actinin[327-329] can affect the rate or extent of polymerization. In addition to these muscle proteins, the recent emphasis on nonmuscle actins has led to the discovery of an ever-increasing list of nonmuscle intracellular proteins involved in actin assembly. These include proteins that affect either the nucleation (e.g., profilin)[330] or the elongation stage (e.g., capping protein)[331] of filament formation and may or may not be sensitive to intracellular Ca^{2+} concentrations. Several recent reviews have provided compilations of these agents and the mechanisms of their actions on actin. In many instances, a variety of effects may be understood in terms of a factor's preferential binding to the pointed or barbed end of the filaments (compare References 34, 35, and 332 to 334).

B. Functionally Important Amino Acid Residues in Actin

With the availability of the amino acid sequence of actin,[274] it has become possible to localize those residues that may play a functional role in actin structure and dynamics. SH groups are essential both for ATP binding and polymerization and for combination with myosin.[335-337] Of the five Cys residues in actin,[338] three can be labeled in the native molecule[339] without affecting its polymerizability or ATP-binding characteristics. Of those, Cys 373, adjacent to the COOH-terminal phenylalanyl residue, is the most reactive. In addition to reacting selectively with Cu^{2+},[340] it can be labeled with a variety

of SH-directed optical[324,341-343a] or spin probes[344-347] that can serve as indicators of either conformational changes in the protein[344-347] or of interactions between actin and other muscle proteins.[324,341-343,347] The recent work of Tao and Cho,[341] for example, in which acrylamide is used to quench the fluorescence of Cys-373-bound IAEDANS has provided evidence that this thiol group is located in a region of the molecule that is partially covered in the F-form of actin as well as by the addition of tropomyosin. The above-mentioned studies of Taylor et al.[324] utilize donor and acceptor molecules attached to this residue to study intersubunit energy transfer in actin filaments. The reactivity of Cys 373 increases upon the combination of actin with HMM subfragment 1, suggesting either the proximity of the group to the myosin heads or at least some coupling between the region of actin containing Cys 373 and that directly involved in myosin binding.[348]

Another residue in actin that functions as an internal probe is Tyr 69, located near the single *N*-methylhistidine residue in the protein. Tyr 69 is selectively nitrated by tetranitromethane, and changes in the absorption of light at 425 nm by the resulting nitrotyrosyl residue suggest that it may be somehow involved in the polymerization process.[338] In other studies, the fluorescent ATP analog, ε-ATP, has been attached to the nucleotide binding site on the protein for energy transfer measurements.

A recent study by Lu and Szilagyi[350] has provided insight into the surfaces of the protein involved in polymerization. They measured differences in the reactivities of the various lysines on the protein surface in F- vs. G-actin and found that polymerization reduced the reactivities of several, viz., Lys 61, 68, 113, and 283. The decrease in reactivity can be explained if the affected residues are located in the area of contact between monomers, although one cannot rule out conformational changes in the protein. An intriguing observation in this work was that Lys 335 exhibited a substantial increase in reactivity in the polymerized form of actin, although it decreased again if actin was complexed with either myosin subfragment-1 or tropomyosin, It would be of interest to determine whether this increased reactivity in Lys 335 reflected a conformational change in actin induced by its incorporation into the filament or whether it might occur upon addition of KCl to actin at a subcritical concentration.

V. INTERACTIONS AMONG THE THIN FILAMENT PROTEINS

A. Troponin Intersubunit Interactions

The regions in the primary structures of the three troponin subunits that are believed to be sites of subunit-subunit interaction are summarized in Table 6. Much of our current information on the details of the assembly of the troponin complex comes from studies on binary complexes. Major findings concerning the properties of these complexes are treated below.

1. TnC-TnI

Formation of the TnC-TnI complex requires Ca^{2+}. The need, in vitro, for a reducing agent such as 2-mercaptoethanol or dithiothreitol suggests that in the native state the thiol groups are in the reduced form. If the complexation is carried out in the presence of 6 *M* urea followed by its removal by dialysis,[59] the final product does not seem different from that obtained in the absence of urea. Under nondenaturing conditions, the complex, once formed, is stable whether or not Ca^{2+} is present.[110] If Ca^{2+} is present, the complex is not dissociated even in solutions containing up to 8 *M* urea as demonstrated by gel electrophoresis.[110] This apparent stabilization of the complex in urea by Ca^{2+}, consistent with earlier studies of Perry et al.,[171] formed the basis for suggestions by Margossian and Cohen,[204] and by Ohnishi et al.[351] that Ca^{2+} may "tighten" the TnC-TnI interaction in the native state as well during regulation.

Table 6
SITES OF INTERACTIONS BETWEEN TROPONIN SUBUNITS

Subunit bound	Putative binding regions[a] (amino acid residues) TnC	TnI	TnT
TnC		1—47[b178]	176—230[b198,200]
		101—113[178,194,195]	
TnI	50—60[b109,169]		1—70[210]
	90—100[b109,168,169]		152—175[198]
	126—136[169]		
TnT	1—100[b169,170]	40—78[209]	
	121—159[109,170]		

Note: Superscript numbers are references.

[a] In many cases the actual site of interaction is within the stretch of residues corresponding to a characterized peptide.
[b] Requires Ca^{2+}.
[c] Based on Lys reactivities in the TnI-TnT complex.

Several more recent studies also point to stronger binding between TnC and TnI in the presence of Ca^{2+} than in its absence.

1. The addition of Ca^{2+} to the high affinity sites of the complex decreases the quenching by acrylamide of a fluorescent probe introduced into a known site of interaction between the two proteins (viz., Cys 98 of TnC).[352]
2. Cross-linking of TnI to TnC by a heterobifunctional cross-linking reagent attached at one end to thiol groups on either TnI or TnC is increased by Ca^{2+}.[207,353](It should be noted that changes in the extent of cross-linking may also result from conformational changes brought about by Ca^{2+} in either protein that increase the number of potentially reacting side chains rather than by stronger binding per se.)
3. Extensive washing of myofibrils with an EDTA-containing solution removes TnC.[354] The extraction is, however, prevented by the inclusion in the wash solution of either Ca^{2+} or Mg^{2+} in concentrations sufficient to saturate the high affinity sites of the TnC.[355]
4. Grabarek et al.[356] have used a kinetic approach to estimate the TnC-TnI binding constant. They measured the rate of exchange of unlabeled TnC with fluorescently labeled TnC in complex with TnI. Addition of the unlabeled protein to the complex resulted in a fluorecence change attributable to the dissociation of the labeled TnC from the complex. The dissociation was more rapid in the absence of Ca^{2+} ($k = 0.07\ sec^{-1}$) than in its presence ($k = 0.006\ sec^{-1}$), a finding consistent with the stabilizing effect of the cation. If one assumes a diffusion-limited on-rate for complex formation in either case (viz., $\sim 10^8\ sec^{-1}\ M^{-1}$), the equilibrium constants for the TnC-TnI interactions in the absence and presence of Ca^{2+} would be 10^9 and $10^{10}\ M^{-1}$, respectively.

Since either Ca^{2+} or Mg^{2+} (in the ternary complex) enhances the stability of the complex, it seems that it is the high affinity Ca^{2+}-Mg^{2+} sites that are involved. This further

implies that the TnC-TnI interaction is always strong in vivo insofar as these sites are always saturated with either Mg^{2+} or Ca^{2+}. In terms of the attachment sites between the two proteins (see Table 6 and section on TnC), these studies suggest that in resting muscle (low cytoplasmic Ca^{2+}), TnC has a two-pronged attachment to TnI involving the Ca^{2+}-insensitive region of residues 126 to 136 and the Ca^{2+}- (and, presumably Mg^{2+}-) dependent site at residues 90 to 100 of TnC and 101 to 113 of TnI.[169,178,194,195] The only remaining site of interaction between the two proteins, involving residues 50 to 60 of TnC and 1 to 47 of TnI, must then become effective only during activation of the muscle.

Corresponding to the enhancement by Ca^{2+} of the binding of TnC to TnI, there is, as might be expected, an increase in the Ca^{2+} and Mg^{2+} binding affinities in the complex as compared with TnC alone.[73] There is, in effect, only a single class of sites in the complex with respect to their affinities depending on [Mg^{2+}] (see Table 2). This, however, is not to be construed as suggesting that all four become simultaneously occupied by Ca^{2+} when muscle is activated. Binding of Ca^{2+} to the high affinity sites in vivo could only occur after Mg^{2+} dissociates, a relatively slow event ($k = 8\ sec^{-1}$) compared to the diffusion-controlled binding of Ca^{2+} to an unoccupied site.[152]

In as much as the activating effect of Ca^{2+} involves a change in TnC that is somehow transmitted to TnI leading to the latter's release from actin and tropomyosin, a key problem that awaits solution is the exact nature of the information transfer between the subunits. Large conformational changes in TnI are ruled out by studies showing that Ca^{2+}-induced changes in circular dichroism in the TnC-TnI complex are only slightly greater than the sum of those in the separate subunits.[122] Several optical and spin probes on TnI complexed to TnC respond to Ca^{2+} binding.[351,357-361] One in particular, IAEDANS, shows spectral changes that occur upon the binding of Ca^{2+} to the low affinity Ca^{2+}-specific sites of TnC, without any change in the far UV circular dichroism.[357] Thus the extrinsic probes report changes in their immediate environments induced by Ca^{2+}; whether this reflects structural changes in TnI per se or whether changes occur on the surface of TnI near the probe owing to altered interactions with TnC is as yet unresolved.

2. TnC-TnT

A large number of studies have independently demonstrated the formation of a binary complex between TnC and TnT,[60,61,169,170,204] although the exact conditions necessary for complex formation differ from group to group. Several reports[61,170,204] indicate that high Ca^{2+} concentrations (>0.1 m*M*) are necessary for the two proteins to interact, while according to others,[169] Ca^{2+} is not essential for complex stability. Relevant to this controversy is the idea that if complex formation was heavily dependent on Ca^{2+} levels, then one might expect that the complex, once formed, would bind Ca^{2+} with an affinity higher than TnC alone, as is the case in the TnC-TnI complex. This has recently been shown to be the case for the TnC-TnT complex in our laboratory (unpublished).

In spite of the unequivocal evidence that TnT can bind to TnC in binary complexes, the extent to which TnT directly interacts with TnC in the ternary complex is unclear. The lysine reactivity studies of Hitchcock,[141] for example, indicate that the lysine residues of TnC that exhibit reduced reactivities in the TnC-TnT complex are the same ones in which reactivities are reduced in the TnC-TnI complex. Furthermore, addition of TnT to a TnC-TnI complex does not change the pattern of lysine reactivities found in the binary complex. Other studies also suggest limited TnC-TnT contact:

1. Leavis and Tao[352] showed that the reduction in solute quenching of a fluorescent probe attached to Cys 98 on TnC was nearly the same for the TnC-TnI and TnC-

TnT complex, suggesting that TnI and TnT could occupy the same binding site on TnC in the absence of the third subunit. Again, however, formation of the ternary complex did not further increase the quenching.

2. A variety of cross-linking studies carried out on whole troponin using either non-specific cross-linkers[206,362] or a specific heterobifunctional reagent, with one attachment at a thiol group,[207] failed to demonstrate more than marginal TnC-TnT linking, even though significant cross-linking of TnC-TnI and TnI-Tn occurred.
3. Grabarek et al.[356] have recently shown that the rates at which TnC exchanges with labeled TnC complexed with either TnI or TnI-TnT are essentially the same, which suggests that TnT does not substantially change the binding strength of TnC to the complex.
5. Finally, the Ca^{2+} binding affinity of the TnC-TnI-TnT complex is identical to that of the TnC-TnI complex, indicating that TnT does not produce detectable changes in the properties of the TnC subunit.[73]

Notwithstanding the above considerations, a role for the TnC-TnT interaction in the ternary complex is suggested by other studies. Ca^{2+}-dependent changes in the tryptophan fluorescence of TnT complexed to TnC have been recently demonstrated in this laboratory (unpublished), suggesting the possibility of direct transfer of structural changes between the two proteins, in agreement with earlier studies using dansyl chloride.[361] Also, Ca^{2+} weakens the binding of the T_2 fragment of TnT to tropomyosin as indicated by affinity chromatography[363] and cross-linking studies[364] when TnC or TnC-TnI is present in the system.

It must be remembered that the Ca^{2+}-dependent, reversible regulation of actomyosin ATPase activity is possible only when all three troponin subunits are present in the system. In a system containing all of the thin filament proteins excepting TnT, the ATPase activity cannot be shut off.[168,169] Thus TnT plays a crucial role in thin filament regulation. However, whether it exerts its effects through a direct but perhaps weak interaction with TnC or indirectly through its interaction with TnI remains to be resolved.

3. TnI-TnT

Although interaction between TnI and TnT was suggested by the composition of troponin B,[53,55] or inhibitory factor,[52] and by the paracrystal studies of tropomyosin containing the two subunits,[204] details of their binding were largely ignored, perhaps owing to their relatively low solubilities in dilute salt solutions. The question was reopened in 1975 when Hitchcock's cross-linking studies[206] suggested that the two proteins were within 0.6 nm of each other in whole troponin. Katayama,[365] using affinity chromatography, and Horwitz et al.,[176] using circular dichroism and gel filtration, demonstrated that TnT and TnI formed a 1:1 complex in KCl solutions ranging from 0.15 to 0.5 *M*. The circular dichroism of the complex, when compared with the sum of the spectra from the individual components, indicated that complex formation resulted in substantial conformational changes. The oxidation state of the three thiol groups of the TnI moiety (see above) was a critical factor. The complex formed without prior reduction of the thiols exhibited a spectrum that was different from that of the native complex and did not confer Ca^{2+} sensitivity on actomyosin ATPase activity when mixed with the other thin filament proteins. Both the spectrum and the Ca^{2+} sensitivity, however, showed the "native" properties upon reduction of the complex.[176] Hincke et al.[177] have made similar observations for the complex from cardiac muscle.

From an evaluation of the studies on intersubunit interactions in troponin, it currently appears that each of the three proteins interacts with the other two, in some

cases at multiple points of contact. It is clear that strong interactions exist between TnC and TnI and between TnI and TnT and that there are perhaps more tenuous TnC-TnT connections. The conformations of all three subunits in the troponin complex are probably somewhat different from those of the isolated subunits. This has been shown directly for the TnI-TnT complex,[176,177] and in the case of the TnC-TnI complex, it can be inferred from the fact that the Ca^{2+} and Mg^{2+} binding affinities are changed.[73] At present there are no definitive studies that directly demonstrate global conformational changes resulting from Ca^{2+} binding to troponin, although several cross-linking studies indirectly indicate that such changes might take place. These include the observations that Ca^{2+} modulates the extent of intersubunit cross-linking in troponin[207,353,362] and that troponin, once cross-linked with dimethyl suberimidate, loses its capacity to confer Ca^{2+} sensitivity on actomyosin ATPase activity,[206] presumably owing to the inability of the cross-linked protein to undergo conformational change.

B. Actin-Tropomyosin

Hanson and Lowy[16] first suggested that tropomyosin is located in the long-pitched grooves of the double actin helix. The precise position was later shown to be slightly asymmetrical, the tropomyosin molecules actually lying slightly toward the periphery of the filament in closer association with one of the actin strands than the other.[366-368] A considerable body of evidence has accrued in the past decade from X-ray and electron microscopic investigations that tropomyosin is shifted from its peripheral position closer to the groove upon addition to the filaments of Ca^{2+}.[369-374] These structural studies, coupled with biochemical observations that actomyosin ATPase activity is inhibited in the absence of Ca^{2+} and activated in its presence,[10] led to the proposal by several groups that tropomyosin in the peripheral position sterically blocked the binding of myosin to the actin filament[369-371] and contraction was possible only after a shift in the position of tropomyosin. As pointed out by Murray and colleagues,[40] for very fast muscles the shift in position would have to be correspondingly fast; $k \sim 10^3$ sec^{-1}. This could imply that the attachment between tropomyosin and individual actin molecules in the filament is rather weak in spite of the fact that the molecules remain associated even under conditions where free tropomyosin concentrations are extremely low,[375] a situation normally suggestive of strong binding. Alternatively, the binding could be strong but the rates of exchange of tropomyosin between two positions on the actin surface could be rapid.

The light scattering studies of Wegner[375] have contributed to our understanding of these interactions. At 38°C, in solutions containing 0.1 *M* KCl and >1 m*M* Mg^{2+}, he obtained highly cooperative binding of tropomyosin to actin. The curves were analyzed in terms of equations developed by McGhee and von Hippel[376] for cooperative binding of large ligands to a one-dimensional lattice with overlapping binding sites and assumed three types of tropomyosin binding to actin:

1. Binding of isolated tropomyosin to seven adjacent actin monomer units along the filament (there are no neighboring tropomyosins for head-to-tail interactions).
2. The bound tropomyosin has one bound neighbor.
3. The bound tropomyosin interacts with two neighboring tropomyosin molecules, one on each side.

In this scheme, the equilibria in question can be defined using two constants, K_1, for binding of an isolated tropomyosin to an actin heptamer, and ω, defined so that $e^{-\omega RT}$ is the free energy difference between singly bound tropomyosin and tropomyosin interacting with one neighbor. Thus the binding constant for type 2 binding, K_2, is the product of the two, viz. $K_2 = \omega K_1$; for type 3 binding, $K_3 = \omega^2 K_1$. Wegner's

analysis of his binding studies yields, for the experimental conditions, $K_1 = 1$ to 3×10^3 M^{-1}, $\omega = 0.7$ to 1.5×10^3 M^{-1}. While the binding of isolated tropomyosin to the actin heptamer is, as expected, rather weak, the overall association of the two proteins is considerably stronger, owing to the interactions between contiguous tropomyosin molecules, viz., $\omega K_1 \sim 2 \times 10^6$ M^{-1} and $\omega^2 K_1 \sim 2 \times 10^9$ M^{-1}. Thus the tropomyosin molecules on the actin filament may be viewed as being strongly attached as a result of their head-to-tail continuity. The importance of the head-to-tail contact in maintaining the integrity of the complex is underscored by the results of recent experiments in which carboxypeptidase A-digested tropomyosin is shown to bind only weakly[377,377a] or not at all[268] to F-actin (the digestion cleaves several residues from the COOH-terminus of the molecule eliminating the head-to-tail interaction). A number of other workers have previously reported equilibrium constants for tropomyosin-actin interaction.[217,378-380,431] It appears that in these cases the reported values correspond to association that involves both isolated tropomyosin-actin plus the head-to-tail binding to a contiguous molecule, i.e., K_2 and K_3.

A number of physicochemical factors influence the strength of the tropomyosin-actin interaction. Generally speaking, conditions that favor the depolymerization of actin also tend to dissociate tropomyosin. Low salt solutions, for example, used in the purification of actin ([KCl] < 0.01 *M*) also decrease tropomyosin binding.[217] It is, however, also decreased by high salt, viz., ;≥ 0.6 *M*.[381,382] The concentrations most favoring interaction range from 0.1 to 0.3 *M*. In the case of divalent cations, Wegner[375] has shown that a fivefold increase in $MgCl_2$, from 0.5 to 2.5 m*M*, doubled the values of K_1 and ω, although most of the increase in K_1 occurred between 0.5 and 1.5 m*M* $MgCl_2$ and that of ω between 1.5 and 2.5 m*M* $MgCl_2$, the significance of which is not clear. Tropomyosin-actin binding is also influenced by the temperature of the medium, the affinity decreasing with increasing temperature particularly above 40°C.[375,383] It is of course necessary to consider all of these conditions when comparing published binding constants between these two proteins.

In addition to the salt and temperature, the nature of the tropomyosin itself affects its affinity for actin. Walsh and Wegner[384] have shown that the equilibrium constant for tropomyosin cross-linked by disulfide bond formation between the two Cys 190 residues is decreased by a factor of two compared to uncross-linked tropomyosin. These authors make the point that, although the difference in the constant is small, the highly cooperative nature of the binding under physiological conditions is sufficient to shift the filament from a nearly completely covered to a nearly completely uncovered state. Although no substantial differences in binding exist between the α- and β-isomorphs of tropomyosin from skeletal muscle,[385]tropomyosin isolated from equine platelets[386] binds poorly to muscle actin, most probably owing to the deletion of 37 residues from its NH_2-terminal end which appears to decrease its head-to-tail interactions.[387]

Several of the other contractile proteins have also been reported to affect tropomyosin binding to actin. Eaton[388] and others.[72] have shown that at low KCl or Mg^{2+} concentrations where binding is normally weak, the addition of heavy meromyosin (HMM) or subfragment-1 to the system induced tropomyosin binding to actin, an unexpected observation in light of earlier views of competition between the two proteins for the same actin binding site. (The reciprocal phenomenon, viz., the induction of HMM binding to actin by tropomyosin, had been reported earlier by Bremel and Weber.[72]) The binding of tropomyosin containing whole troponin (TnT+TnI+TnC) or the binary complex TnT-TnI was recently studied by Wegner and Walsh[389] using an equilibrium scheme similar to that they employed for the binding of pure tropomyosin.[375] They found that both troponin-tropomyosin, in the absence of Ca^{2+}, and TnT-TnI-tropomyosin have an affinity for F-actin which is about ten times higher than that of

pure tropomyosin in the isolated type of association ($K_1 \sim 1.5$ to $1.8 \times 10^4\ M^{-1}$) and about 200 to 300 times higher in the single contiguous ligand type of binding ($K_2 \sim 4 \times 10^6\ M^{-1}$). Ca^{2+} decreased the affinity of the troponin-tropomyosin complex for actin by more than a factor of two ($K_1 \sim 6600\ M^{-1}$), while the value of ω, the head-to-tail interaction parameter, remained unchanged. Finally, using a mixture of troponin-tropomyosin-F-actin and TnT-TnI-tropomyosin-F-actin in presence of Ca^{2+} (this mixture is expected to contain tropomyosin molecules both in the $-Ca^{2+}$ and $+Ca^{2+}$ positions on the actin surface), Wegner and Walsh[389] found that the binding constant of adjacent tropomyosin molecules in different positions on the filament is about one half that for adjacent molecules in the same position.

The increased tropomyosin-actin affinity induced by troponin or TnT-TnI as described above may result primarily from the interactions of the TnI component. This subunit is well-known to interact with both actin[172,390] and TnT[176,177,365] and via the latter's attachment to tropomyosin ties the four molecules—actin, tropomyosin, TnT, and TnI—together. It also appears that TnI may also directly bind to the tropomyosin; this follows from the old observation that TnI alone could inhibit pure actin from interacting with myosin and that this inhibition could be enhanced by the addition of tropomyosin[60] and from more recent direct measurements of TnI-tropomyosin interaction using affinity chromatography.[391] That TnI may directly cross-link tropomyosin to actin is further suggested by the demonstration by Cote and Smillie[386] that pure TnI induced the binding of platelet tropomyosin to muscle actin. These considerations, coupled with the well-established observation that the TnI-actin bond is weakened by TnC,[172,204,390] particularly in the presence of Ca^{2+}, and that the hydrolysis of ATP is at the same time uninhibited, provide compelling evidence for the view that troponin, in the absence of Ca^{2+}, acting principally through the TnI subunit, stabilizes the tropomyosin-actin interaction in the "off" state.

C. Overall Assembly of the Thin Filament Proteins

There are extensive interactions between the five constituent proteins of the thin filament (Figure 21a). The intersubunit interactions of troponin have been documented above as have those between tropomyosin and actin. The additional connections linking each troponin complex to actin-tropomyosin were originally viewed in terms of two-site binding in which the TnI moiety was bound to actin and the TnT moiety to tropomyosin.[60,172,392-394] The question of a direct TnI-tropomyosin link suggested by early observations that a TnC-TnI complex could bind to actin-tropomyosin, but not to actin alone[172,393] and that TnI enhanced tropomyosin binding to actin[395] was unequivocally settled by the demonstration that TnI could bind to a tropomyosin affinity column.[391] In Figure 21a we have left out a direct connection between TnT and actin, although it had been suggested by past studies carried out under nonphysiological conditions[396,397] or when a modified actin was studied.[398] Katayama[391] has pointed out that the α-helical portion of the T_1 fragment of TnT exhibits patterns of repeating charged residues similar to those of tropomyosin which may account for its ability to mimic the latter with respect to actin binding.

The addition of Ca^{2+} to complexes of the thin filament proteins appears to affect the properties of almost all of its interprotein interactions (Figure 21b). The strengthening of the tie between TnC and TnI has been noted in many studies[110,172,204,393] and is likely to involve Ca^{2+}-induced binding between loci in the amino acid sequences of the two proteins in addition to the sites of attachment that exist in the absence of Ca^{2+} (see above). The increased TnC-TnT interaction has also been mentioned, although the precise nature of this change is unknown. The weakening of the bond between TnT and tropomyosin indicated by the dashed line is suggested by the recent work of Pearlstone and Smillie[363] who have shown that the T_2 peptide of TnT can be dissociated

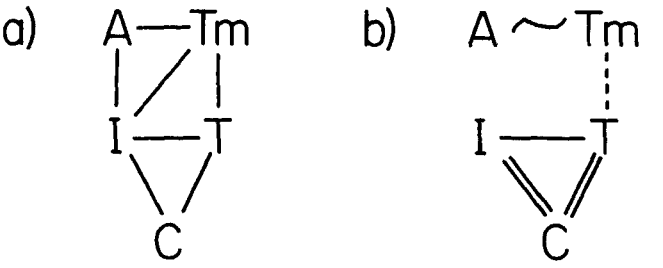

FIGURE 21. (a) Diagram showing the intrathin-filament interactions in the absence of Ca^{2+}. A = actin; Tm = tropomyosin; T = TnT; I = TnI; C = TnC. (b) In the presence of Ca^{2+} virtually all interactions are modified, i.e., bonds involving TnC with TnI and TnT are strengthened while those of TnI to actin and tropomyosin and of TnT to tropomyosin are either weakened or abolished (see text). The tropomyosin-actin interaction changes as is indicated by both structural and biochemical studies (see section VI).

from a column of immobilized tropomyosin by the addition of TnC or TnC-TnI in the presence of Ca^{2+}. Finally, the curved line between the actin and tropomyosin indicates the still poorly understood difference in their interaction in the activated and inhibited state.

Although it seems reasonable that the various changes in the interactions among the thin filament proteins result from, or are at least accompanied by, changes in the conformation of a number of them, demonstrations of such changes are few. With regard to troponin itself, fluorescent probes specifically attached to sites on TnC[399] and TnI[360] in the thin filament exhibit spectral changes upon Ca^{2+} binding interpretable as changes induced in TnC that are somehow transmitted to the TnI component. Ca^{2+} also seems to modulate the extent of cross-linking by various agents of TnC and TnI[207,353,362] and TnC and TnT[170] and to affect the number of surface NH_2-groups available for chemical modification in troponin.[141,192,193,209,362]

Tonomura et al,[400] using electron spin resonance, and more recently, Wahl et al.,[257] employing anisotropy decay of a tropomyosin-bound probe, have presented evidence that the flexibility of tropomyosin complexed to troponin is dependent upon Ca^{2+} in the micromolar range. How this relates, if at all, to conformational changes in the molecule, such as the local unfolding described previously, remains unresolved. In the case of actin, structural modifications brought about by Ca^{2+}-binding to the filament are suggested by changes in the linear ultraviolet dichroism.[401,402] Although it is outside the purview of this review, it bears mentioning that several studies have demonstrated that myosin subfragment 1 binding to actin induces conformational changes in the latter (e.g., References 403, 404).

A number of cross-linking studies indicate that such Ca^{2+}-induced conformational changes as do occur in the thin filament proteins appear to be necessary in the regulatory mechanism. Hitchcock[206] showed that isolated troponin cross-linked with various dimethylimido esters lost the ability, when incorporated into the thin filament, to confer Ca^{2+} sensitivity on the actomyosin ATPase activity, although it retained its ability to bind to actin-tropomyosin and to Ca^{2+}. In other studies,[380,405,406] glutaraldehyde-cross-linked actin[406a] was shown to activate the ATPase activity of myosin subfragment-1, but the activation could not be controlled by addition of troponin-tropomyosin to the system. This was interpreted to mean that the cross-linked actin was frozen in an active conformation. This further implies that the action of troponin-tropomyosin in the native system must in part involve the induction of a change in the structure of actin. Finally, Mikawa[407] used glutaraldehyde to cross-link the complete thin filament. If the cross-linking was carried out in the presence of Ca^{2+}, the system was frozen in the "on" state — viz., actomyosin ATPase was activated even in the

absence of Ca^{2+} — whereas when EGTA was present, the system permanently remained in the inhibited state.

No correlation has yet been made between the conformational changes suggested by these studies, carried out on proteins in solution, and the change in the azimuthal angle of the troponin-tropomyosin complex with respect to a projection of the filament that links two nearest-neighbor actin monomers on a plane perpendicular to the filament axis as described in the earlier structural studies.[366-374] The application to this problem of fluorescence energy transfer in which donor and acceptor fluorophors are attached to components of the regulatory complex and to actin (or vice versa), respectively, may be promising for the detection of such a change in solution. This will require, however, the successful solution of complications arising from the stoichiometry of the thin filament proteins that results in a 1:7 ratio of donor to acceptor or acceptor to donor. It has also been calculated by Tao and Morris[408] that, depending on the locations of the probes, substantial movement of tropomyosin, could take place over the actin surface without significant effects on the energy transfer efficiency. A Ca^{2+}-dependent change in the transfer efficiency between an actin-bound donor and an acceptor on TnC has been reported.[409]

VI. MECHANISM OF REGULATION

By the early 1970s X-ray diffraction studies, as well as analysis of light diffraction of electron micrographs, suggested differences in the thin filament structure of relaxed, rigor, and activated muscles. These led to what became known as the steric blocking model of regulation. Before we discuss it and more recent developments concerning the mechanism of regulation, a discussion of the structural aspects seems in order.

A. Structural Aspects of Regulation

In 1963, before troponin was discovered and before a functional role for tropomyosin was found, Hanson and Lowy[16] had suggested that tropomyosin might lie in the long-pitch groove of the actin filaments. This proposal acquired more substance when Moore et al., applying three-dimensional reconstruction techniques to electron micrographs, showed the presence of additional material, compared with pure actin filaments, in the long-pitched grooves.[366] Also taking into account developments relating to the biochemistry of the tropomyosin-tropinin system, they suggested that this material may correspond to the tropomyosin-troponin complex. Analysis of X-ray diffraction patterns from live vertebrate muscle showed that in the resting state the second actin layer line is absent and the third layer line is visible while in contracting muscle the second layer line is stronger than the third. These results were interpreted[369-371,410] as being due to the movement of tropomyosin from a position in contact with one of the two strands of actin monomers in resting muscle to a more central position in the long-pitch helical groove in activated muscle (Figure 22a). Lowy and Vibert[411] found similar transitions in molluscan muscle, although in that case regulation is based on the interaction of Ca^{2+} with myosin.

Spudich et al.,[368] working with reconstituted thin filaments, demonstrated that the extra density seen in three-dimensional image reconstructions was indeed due to the presence of regulatory proteins and was located asymmetrically in relation to the groove. Wakabayashi et al.[372] found that there was a difference in the position of the density corresponding to tropomyosin if one compares the actin-tropomyosin system with actin-tropomyosin-TnI. They reported the tropomyosin-actin system as the equivalent of the active state and the actin-tropomyosin-TnI system as that of the inhibitory state; in the latter, the extra density was even more asymmetrically placed with respect to the central groove than in the active state.

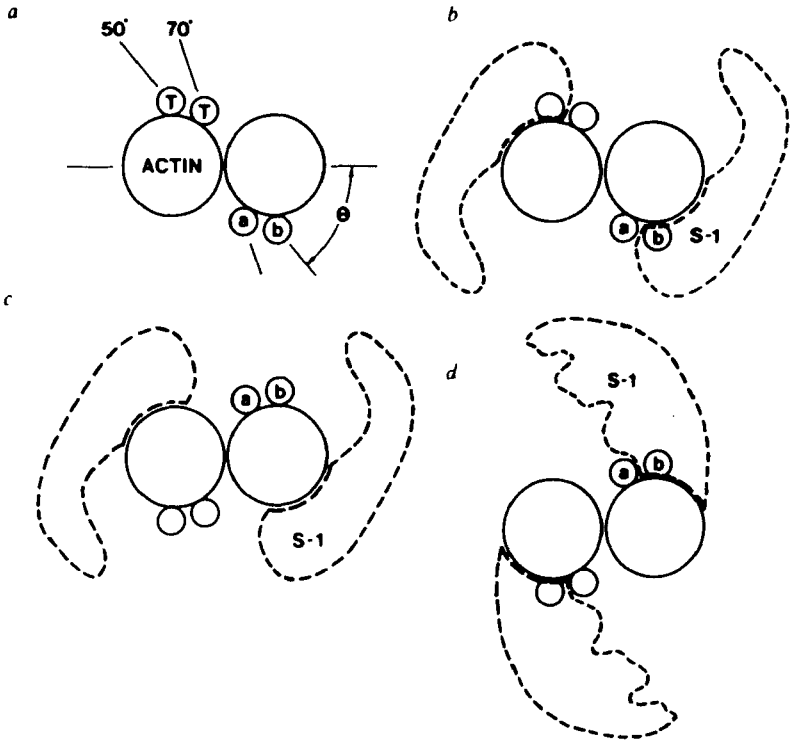

FIGURE 22. Schematic illustrations of the evolution of the regulation story (see Reference 42). (a) View looking down the thin filament axis towards the Z-line showing the two actin strands (assumed continuous for ease of representation) and the two tropomyosin positions (T, $\theta = 50°$ and $\theta = 70°$) deduced from X-ray diffraction data.[367-372] Note that these data also allow the tropomyosin positions to be bottom left and top right in contrast to the arrangement shown. (b) The myosin head (S-1) attachment position according to Moore et al.[366] is close to tropomyosin position 'b' (the blocking position; $\theta = 50°$), but it is away from position 'a' (the active position, $\theta = 70°$). (c) Seymour and O'Brien[373] concluded that tropomyosin is on the opposite side of the actin strand from Moore et al.'s myosin head position. (d) Reconstruction of decorated thin filaments by Taylor and Amos[416] place the myosin on the same side with tropomyosin, being counter-clockwise from the groove so that steric blocking can still occur. It would be difficult to envisage steric blocking with (c), but possible with (b) or (d). (Reprinted by permission from Squire, J., *Nature*, 291, 614, Copyright © 1982, Macmillan Journals Limited.)

A comparison of the suggested location of the subfragment 1 binding on the actin monomers, deduced from studies on thin filaments decorated with subfragment 1,[366] with the proposed location of tropomyosin in resting muscle led to the conclusion that in relaxed muscle tropomyosin and subfragment 1 would occupy the same site on actin (Figure 22b).[369-371] This is the basis for the steric blocking model for regulation. The calcium-induced movement of tropomyosin, it was thought, would lead to vacating of the site and would thus permit the binding of subfragment 1. As pointed out by O'Brien and colleagues,[412] while the azimuthal shift in the position of tropomyosin could unequivocally be deduced from the diffraction data, the relation of the binding sites of tropomyosin and subfragment 1 cannot be determined without ambiquity unless the polarity of the actin filaments was the same as in experiments[266] from which the subfragment 1 position has been deduced. Seymour and O'Brien[413] have turned to the use of thin filaments attached to Z lines, in which polarity[414] can be compared with that of decorated filaments for three-dimensional image reconstruction. Their results indicate that tropomyosin in the thin filament is located on the opposite side of the groove from the position of subfragment 1. This can be best described by saying that if one looks down the thin filament towards the Z line (Figure 22c), for a given actin

monomer subfragment 1 would be in a clockwise position from the equator and the tropomyosin in a counterclockwise position.

O'Brien and colleagues,[412] while agreeing with other workers on the direction of the azimuthal movement of tropomyosin, place more emphasis on the radial changes in the density than they ascribe to changes in the position of tropomyosin. They suggest that under conditions corresponding to activation, tropomyosin is closer to the groove but at a higher radial distance, while in the inhibited position, it is closer to the edge of the thin filament but more integrated with the actin structure and hence at a lower radial position. It also appeared that Ca^{2+} induces changes in actin paracrystals in the helical symmetry of the actin filament. However, it turns out that these differences appear to be related to the packing of the filaments and to whether they are parallel or antiparallel rather than to the state of activity. Thus the presence of tropomyosin and TnT in the presence of TnI results in an inhibited filament in which changes in helical symmetry do not occur, and the key feature that can be related to activity is the position of tropomyosin rather than parameters of the actin helix. The recent work of Egelman et al.,[415] showing the presence of a variable twist in actin filaments and indicating differences between filaments in paracrystals and in the native state, suggests that interpreting results derived from paracrystals, where the packing of filaments may be a dominant feature in terms of actin symmetry, may be rather risky.

More recently, Taylor and Amos[416] reexamined the problem of actin-myosin-tropomyosin interaction by improved techniques for which the reader is referred to the original work. They obtained three-dimensional reconstructed images of native thin filaments and of purified actin decorated with rabbit myosin subfragment 1. Their results show significant differences from the reconstructed images reported by Moore et al.[366] and suggest that actin is located closer to the filament axis, compared with the earlier work, and features of the density pattern previously assigned to subfragment 1 would be attributable to actin. Although the precise location of tropomyosin is still somewhat conjectural, as is the case in Wakabayashi's analysis,[372,417] they agree with O'Brien et al.[412] in placing tropomyosin in the counterclockwise position from actin looking toward the Z axis. However, they also place the bulk of subfragment 1 in that quadrant, thereby reopening the possibility of a steric blocking model (Figure 22d).

Most recent structural information, however, again raises serious questions with regard to the validity of the structural basis of steric blocking. Recent data concerning the shape of actin[418,419] point to the need for possible changes in the detailed interpretation of thin filament structure. Current data agree with those of Moore et al.,[366] suggesting an asymmetric actin monomer; however they point to a more tangential location of the actin monomer axis with respect to the helix rather than the earlier axial orientation. Amos et al.,[420] in an attempt to incorporate the findings of Kassab's group[421] concerning the attachment of each subfragment 1 to two actin monomers, still find a steric blocking mechanism compatible with the three-dimensional reconstruction from thin filament micrographs (Figure 23).

Electron microscopic work on filaments decorated with molluscan heavy meromyosin fibers,[422,423] a system subject to myosin-linked regulation, showing that changes occur near the tips of the arrowheads depending on whether the regulatory chain is present or absent, suggests that the inhibition of actomyosin ATPase activity is not related to the presence of the light chains in a position that might directly change the interaction with actin. While, in view of the differences in actin-filament-regulated and myosin-regulated systems, the force of this conclusion for the former may not be overwhelming, it adds to the body of evidence (discussed by Chantler)[43] that may require us to take a fresh look at the mechanism of activator and inhibitor. The picture grows even more complex as we turn to the biochemical data.

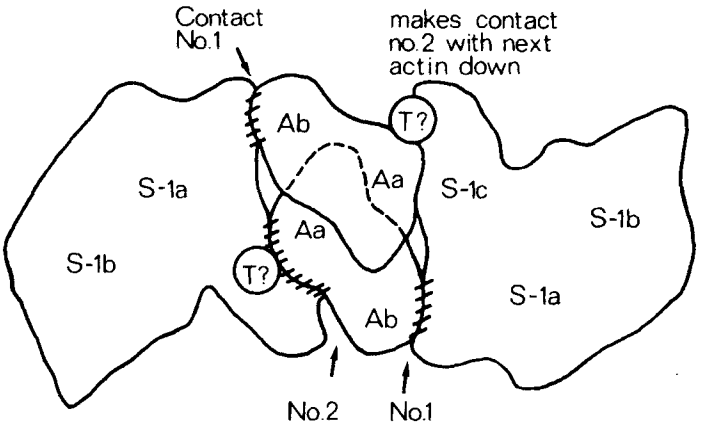

FIGURE 23. Diagram of the proposed interactions in the rigor state between myosin heads (S-1), actin monomers (A) in F-actin and tropomyosin molecules (T) which are thought to lie in the actin helix grooves. This figure is an updated version of Figure 3 of Reference 420 provided by the authors. Distinct sites on each S-1 contact two neighboring actin monomers and there is close contact between S-1 and tropomyosin that may account for the stronger binding of S-1 to regulated actin than to unregulated actin.[388,434] Each actin monomer consists of two stain-excluding domains (Aa and Ab) in agreement with other data;[418] S-1 consists of three such domains (S-1a-c). (Reprinted by permission from Amos, L. A., Huxley, H. E., Holmes, K. C., Goody, R. S. and Taylor, K. A., *Nature*, Vol. 29, 301, Copyright © 1981, Macmillan Journals Limited.)

B. Biochemical Aspect of Regulation

The scheme by which calcium binding to troponin causes a shift in the position of tropomyosin permitting myosin heads to attach to actin offered a ready explanation for the fact that Ca^{2+} binding to a troponin complex attached to a tropomyosin molecule affected all actins within the domain of that tropomyosin. A model that involved tropomyosin influencing several — actually seven — actin monomers also accounted rather well for the observation by Bremel and colleagues[424] that at low MgATP concentrations, actomyosin ATPase increased with the MgATP concentration even in the presence of EGTA. As the ATP concentration was further increased in the absence of Ca^{2+} the ATPase activity decreased, while with Ca^{2+} added it increased to a plateau corresponding to the saturation of myosin with ATP. The model also opened the way to an understanding of cooperative phenomena in the interaction of myosin with regulated actin. When a fraction less than 50% of the actin monomers form rigor complexes with myosin, all the monomers in the filament are turned on; their interaction with myosin produces rapid turnover of the actin-ADP-P-myosin complex.[425] In accordance with current practice we shall refer to actin that is combined with tropomyosin and troponin as regulated, although some phenomena observed within this system are also encountered with actin-tropomyosin without troponin.

The high activity at low ATP concentrations could be explained by the formation of so-called rigor links between myosin heads free of ATP and actin as a result of successful competition with tropomyosin for the common site on actin. Consequently, tropomyosin would be found to change its position and make the actin site available for myosin heads carrying the hydrolysis products of ATP. Combination with actin would lead to an accelerated release of the hydrolysis products (compare Reference 426). As the ATP concentration was increased, fewer rigor links could form and the calcium requirement for activation emerged.

Even at the time of its formulation, the steric blocking model, in its purest form in which it is assumed that in the presence of Ca^{2+} the troponin-tropomyosin system exerts no effect on the actin-myosin interacton, was seen to be at variance with some experimental facts. Bremel et al.[424] showed that activation of myosin ATPase, interpreted at

that time as enhanced binding of myosin by actin, was enhanced by tropomyosin-troponin even in the presence of Ca^{2+}. Weber and colleagues[424] used the concept of potentiation earlier applied by Katz[427] to the activating effect of tropomyosin to describe this phenomenon. They varied the ATP concentration and found a biphasic effect; there was first an increase in actomyosin ATPase activity followed by a decline at higher ATP levels. This was attributed to an increase in the "cofactor activity" of actin owing to the formation of rigor bridges at low ATP concentration. In contrast to the simple steric blocking model, all of these studies suggested that in the presence of Ca^{2+}, activation was not complete and the system could exist in a state of higher activity, owing to, according to the earlier thinking, increased affinity between myosin and actin after the initial binding of myosin. This was achieved, presumably, through the rigor bridges whose higher affinity could overcome the lower affinity of the first myosin-product complex within a given tropomyosin domain. It should be emphasized that, in contrast to some current proposals, this cooperativity was confined to the domain of a given tropomyosin molecule. Although cooperativity involving myosin heads that underwent an ATPase cycle was suggested by the earlier experiments, more definite evidence has recently been published.[428]

The work of Eaton and colleagues[388] shows rather directly that myosin binding to actin can be enhanced by tropomyosin and, conversely, the binding of tropomyosin is also enhanced by the presence of myosin. They also furnish clear-cut evidence that at low myosin or, actually, subfragment 1 concentration, the presence of tropomyosin inhibits ATPase activity at all ATP concentrations if the myosin to actin ratio is low. At higher myosin to actin ratios, inhibition occurs at saturating ATP concentrations and gives way to activation at lower ATP concentrations, suggesting cooperativity between myosin heads, some of which may be in a rigor state. TnI enhances tropomyosin-actin binding, a finding complementary to that of Potter and Gergely[172] showing that tropomyosin enhances the binding of TnI to F-actin. The inhibitory effect of TnI on the actomyosin ATPase is attributable in part to effects on tropomyosin binding and to modulation of the effect of bound tropomyosin.

Thus tropomyosin can be seen to exert a dual effect — even in the absence of troponin — depending on the concentration. The dual effect of tropomyosin was first reported by Katz,[427] who showed inhibition of actomyosin ATPase activity occurring at high levels of ATP followed by activation as the ATP level drops owing to hydrolysis. Additional reports have confirmed that the effect of tropomyosin may be either inhibitory or activating,[429,430] although the maximum inhibition reported was only about 60%. Recently Lehrer and Morris[431] published an account of their studies under a wider range of conditions showing that at lower myosin subfragment 1 concentrations, the ATPase activity is inhibited in comparison with pure actin — whether Ca^{2+} is present or not — and that at higher myosin subfragment 1 concentrations, the activity exceeds that of the pure actin system (Figure 24). We shall return to the interpretation of these data in the light of a comprehensive theoretical proposal to deal with tropomyosin-troponin inhibition.

Sobieszek, in a recent study,[432] analyzed the inhibitory effect of skeletal tropomyosin on actin-activated myosin ATPase activity. The myosin in this case was represented by subfragment 1 and heavy meromyosin which, being soluble at low ionic strength, permitted a conventional kinetic analysis. He showed that tropomyosin reduced V_{max} while increasing the K_m obtained from a 1/V vs. 1/[actin] plot. Although the precise meaning of K_m for a modifier such as actin in a system containing many kinetic steps cannot be interpreted in terms of a dissociation constant, the simplest interpretation is in terms of an increase in the apparent affinity by a factor of six to ten, again a fact not readily reconcilable with a mechanism such as steric blocking that would lead to a reduced *affinity*.

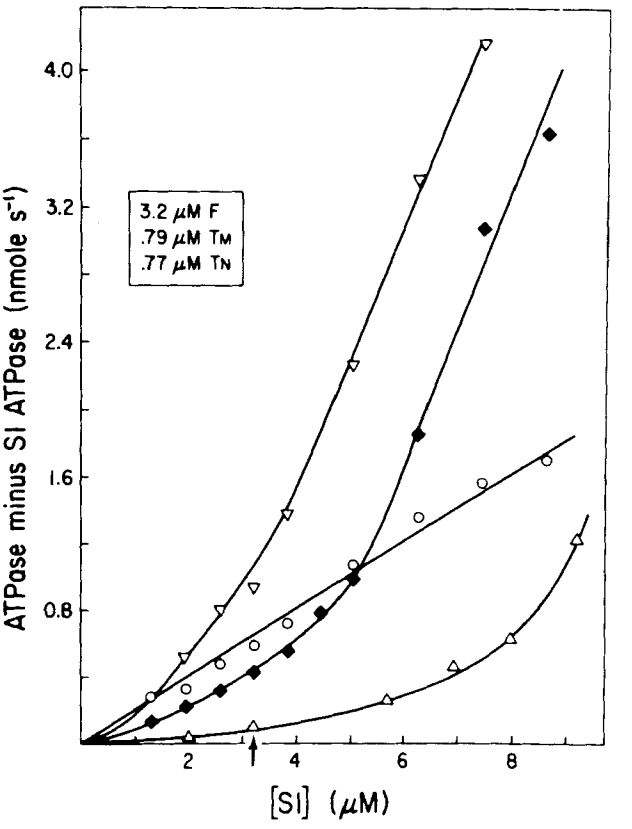

FIGURE 24. The dual effect of the regulatory proteins on the acto-S-1 ATPase activity. The presence of tropomyosin in the acto-S-1 system results in inhibition of ATPase activity at low S-1 (♦) relative to pure actin (○). The tropomyosin-actin curve is shifted by troponin to the left in the presence of Ca^{2+} (▽) and to the right in its absence (△).[431] (From Lehrer, S. S. and Morris, E. P., *J. Biol. Chem.*, 251, 8073, 1982. With permission.)

Analysis of the binding of subfragment 1 to reconstituted actin filaments, viz., actin-tropomyosin-troponin, led to the demonstration of very strong cooperativity among myosin heads in the absence of Ca^{2+}; under these conditions very little binding took place up to about 1 μM free subfragment 1 which was followed by a rather abrupt initiation of binding as the subfragment 1 concentration was increased (Figure 25).[433] The cooperativity was greatly reduced in the presence of Ca^{2+} when the binding occurred at lower subfragment 1 concentrations.

As mentioned above, explanations of cooperativity by Weber and colleagues[424,425] involved binding of several myosin heads within the same tropomyosin domain. In view of there being seven actins in the domain of a tropomyosin molecule, this kind of cooperativity would lead to a seventh power dependence on concentration if the classical Hill plot was used. Greene and Eisenberg[433] found that a Hill coefficient of 60 would be required to come close to their observed cooperativity. Good agreement with the data was obtained with a model,[434] including nearest-neighbor interactions between troponin-tropomyosin units on F-actin without requiring cooperative effects among myosins belonging to different tropomyosin units. The analysis was done in the framework of an infinite one-dimensional Ising model, taking into account the effect of subfragment 1 and calcium binding and utilizing the matrix method. The model postulates that tropomyosin-troponin units can exist in two states characterized by weak and strong binding of myosin, respectively (Figure 26). There are seven actin units

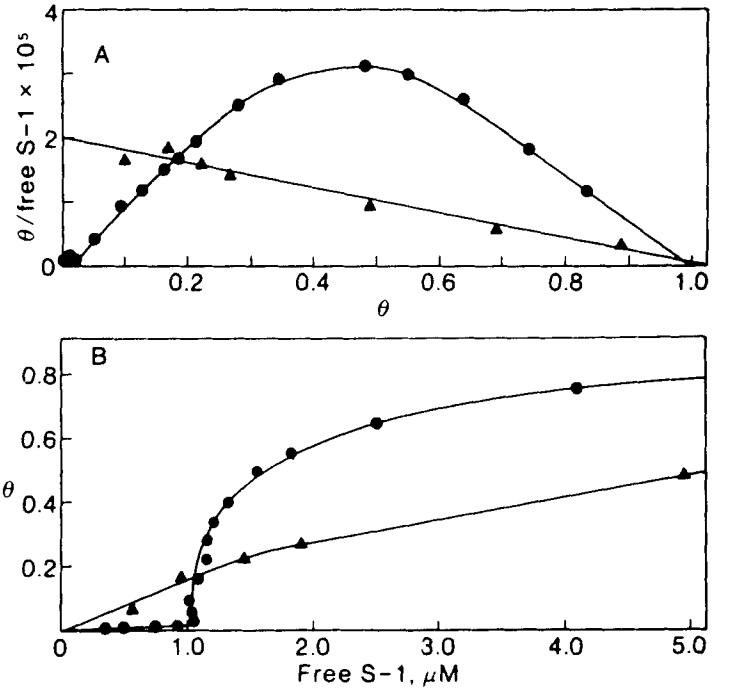

FIGURE 25. (A) Scatchard plot of S-1 binding to regulated (●) and unregulated (▲) actin in the absence of Ca^{2+}.[433] (B) The same data plotted as fraction of actin saturated with S-1 (θ) vs. free S-1. In regulated actin very little binding takes place until the concentration of S-1 reaches ~1 *M* where highly cooperative binding (Hill coefficient ~60) occurs. In the presence of Ca^{2+} (not shown) the cooperativity is reduced (Hill coefficient ~7). (From Greene, L. E. and Eisenberg, E., *Proc. Natl. Acad. Sci. U.S.A.*, 77, 2616, 1980. With permission.)

within the range of one tropomyosin unit which contains a troponin with two metal binding sites. These two sites correspond to the two calcium-specific sites as discussed earlier. Interactions between nearest neighbors are modulated by Ca^{2+} binding to the right-hand member of each pair, corresponding to the asymmetrical location of troponin on tropomyosin (see above). In this model, state 1 is favored at low subfragment 1 concentrations, state 2 at high. Cooperativity is the result of there being seven actin sites within a single troponin-tropomyosin unit and of nearest-neighbor interactions between the units. It is assumed that interactions between neighboring units in the same state are stronger than those between units in different states. Suitable values for the parameters occurring in the model lead to a good fit between theory and experiment. The model permits the calculation of the fraction of actin sites in the strong binding state both as a function of Ca^{2+} and myosin heads. It appears that the weak binding state is favored even in the presence of Ca^{2+} at low myosin concentrations. The switching on, i.e., the favoring of actins in the strong binding state, can be brought about by either Ca^{2+} or myosin or a combination of the two. Thus, in a sense, the system is not turned on uniquely by Ca^{2+} and the question arises what the relation of the structural changes produced by Ca^{2+} in the absence of myosin to the activation of the thin filament is. In terms of the model the structural change observed in the presence of Ca^{2+} alone would essentially correspond to the binding of Ca^{2+} to units in state 1. The structural counterpart of the switching over to state 2 as the result of subfragment 1 binding remains to be resolved.

Evidence for cooperativity also emerges from kinetic studies of the binding of subfragment 1 to pure and regulated actin filaments in the presence and absence of

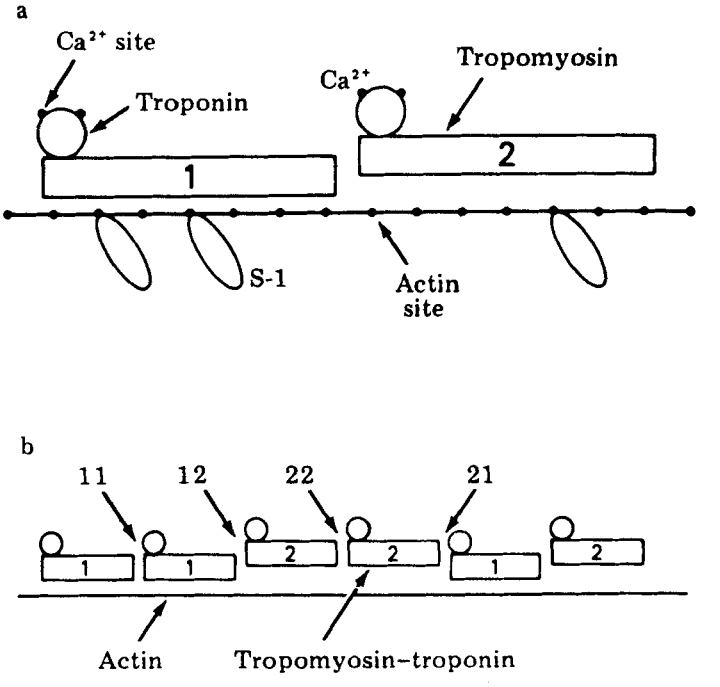

FIGURE 26. (a) Schematic representation of the two states of the troponin-tropomyosin unit which includes seven actin sites. Each troponin has two "regulatory" Ca^{2+}-binding sites. (b) Illustration of the types of nearest-neighbor pairs in a sequence of units.[434] (From Hill, T. L., Eisenberg, E., and Greene, L., *Proc. Natl. Acad. Sci. U.S.A.*, 77, 3186, 1980. With permission.)

Ca^{2+}.[360] Binding monitored by light scattering or by an increase in the fluorescence of a fluorophor attached to the TnI moiety showed cooperativity. A better fit for the binding data was obtained with the Hill et al.[434] two-state model involving nearest-neighbor interactions between tropomyosin units than without such interactions and assuming cooperativity only within the seven-site binding units corresponding to a single tropomyosin molecule. The fraction of sites apparently open for subfragment 1 binding (strong binding sites), deduced from equilibrium measurements, was 10^{-3} in the absence of Ca^{2+}, a value similar to that calculable from the data of Greene and Eisenberg.[433] However, the fraction of open sites inferred from kinetic measurements is close to 15%. Transient kinetic results reveal cooperativity through a lag phase at low but not at higher subfragment 1 concentrations. Interpretation of the kinetic results is complicated by a two-step binding process for pure actin, actin-tropomyosin, and regulated actin plus Ca^{2+}.

The model involving nearest-neighbor interactions between tropomyosin units offers a framework in which the binding of myosin heads to regulated actin filaments can be interpreted while maintaining a cooperative unit involving seven actins in accordance with structural features. It is also possible to fit the data with more or less success by assuming cooperative units of various lengths and having various types of flexibility within the unit.[435] An analysis carried out by Nagashima and Asakura[436] involving the use of a thiol-modified subfragment 1 in competition with unmodified subfragment 1 led to a satisfactory agreement between the binding of these myosin fragments and their effect on ATPase activity without introducing interaction among tropomyosin units, but only by taking values for the number of actin monomers within cooperative units that exceed seven. Weber and colleagues[40,437] have suggested models in which

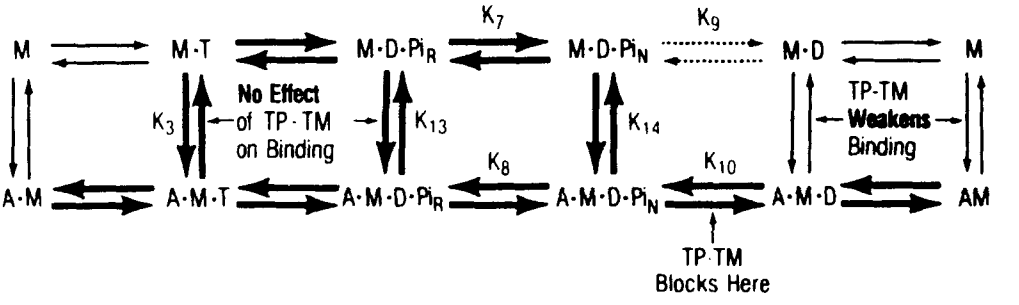

FIGURE 27. Kinetic model of ATP hydrolysis by myosin showing possible step of regulation by troponin-tropomyosin.[441] M = myosin or S-1; A = actin; T = ATP; D = ADP; TP·TM = troponin-tropomyosin; the subscripts R and N are the refractory and nonrefractory states, respectively (see Reference 8). (From Chalovich, J. M. and Eisenberg, E., *J. Biol. Chem.*, 257, 2432, 1982. With permission.)

flexibility within tropomyosin is taken into account and which would explain certain features of binding and ATPase experiments. Flexibility with tropomyosin units has also been proposed to explain certain features of kinetic experiments.[360] The resolution of the discrepancy between kinetics and binding data will require more work.

If one wishes to apply a modeling approach to the analysis of actin-activated steady state myosin or myosin fragment ATPase activity, the situation is more complicated than for equilibrium binding studies. Detailed analysis will require the knowledge of a large number of kinetic constants and the steady state concentrations of various intermediates. A detailed discussion of this problem is beyond the scope of this review. It should be recalled that during the last decade the view introduced by Lymn and Taylor[426] and by Trentham and colleagues[6] has gained general acceptance — that ATP is hydrolyzed on myosin heads and in the absence of actin the dissociation of products is a slow process. The activation of actin is regarded as being due to the acceleration of the release of the products, first phosphate and then ADP, from the myosin heads. In all the preceding discussions of the mechanism by which the troponin-tropomyosin system controls ATPase activity, it was assumed that in some way the interaction of actin and myosin is inhibited, whether or not a simple steric blocking view is correct.

Recently two aspects of the kinetic mechanism have been brought into question by Eisenberg and colleagues. One concerns a return to an older view that prevailed before that of Lymn and Taylor[426] and of Trentham et al.,[6] viz., that hydrolysis of ATP can take place on undissociated actin-myosin complexes.[438,439] This view would increase the complexity of the actomyosin scheme since each kinetic step would have to be considered as taking place both on free myosin and myosin heads complexed with actin.

The second and perhaps more serious complication has to do with recent evidence suggesting that the regulation of actomyosin ATPase activity by Ca^{2+} does not at all involve changes in the fraction of myosin heads dissociated but would involve control of one or more kinetic steps controlling transitions among intermediates representing myosin attached to actin. Chalovich et al.[440,441] have shown that under their experimental conditions, which include rather low ionic strength, subfragment 1 plus regulated actin systems show no Ca^{2+}-dependent change in the fraction of myosin heads bound as deduced from light scattering and sedimentation studies, whereas the ATPase activity is greatly increased upon addition of Ca^{2+}. These observations have prompted Chalovich et al. to suggest that regulation would consist in the control of a kinetic step, most likely that in which phosphate is released (Figure 27).

It would seem that until more information is available on the precise details of the actin-myosin ATPase cycle, modeling may at best be of heuristic value and final judgment on the applicability of a given model has to be withheld. Nevertheless, Hill et

al.[434] have proposed a framework in which the two-state actin filament model developed from binding studies can be adapted to the analysis of ATPase activity. Lehrer and Morris[431] have used this framework to analyze their data on the regulated ATPase activity, as a function of subfragment 1 concentration. So far it appears that the cooperative behavior did require the assumption of a small but definite difference in the binding of the ATP and ADP-P complexes in the two postulated states. They also found that the cooperative feature of the curves did depend in a critical way on the nearest-neighbor interaction parameters. Considerably more work is required on both the theoretical and experimental aspects.

ACKNOWLEDGMENT

The preparation of this manuscript and work in the authors' laboratories are supported by grants from NIH (HL-5949, HL-20464), the National Science Foundation, and the Muscular Dystrophy Association of America, Inc. P.C.L. is an Established Investigator of the American Heart Association.

REFERENCES

1. Engelhardt, W. A. and Ljubimova, M. N., Myosins and adenosinetriphosphatase, *Nature (London)*, 144, 168, 1939.
2. Straub, F. B., Actin, in *Studies from the Institute of Medical Chemistry University of Szeged*, Vol. 2, A. Szent-Gyorgyi, A., Ed., S. Karger, Basel, 1943, 3.
3. Huxley, A. F. and Niedergerke, R., Structural changes in muscle during contraction. Interference microscopy of living muscle fibers, *Nature (London)*, 173, 971, 1954.
4. Hanson J. and Huxley, H. E., The structural basis of contraction in striated muscle, *Symp. Soc. Exp. Biol.*, 9, 228, 1955.
5. Hasselbach, W., Die Umwandlung von Aktomyosin-ATPase in L-myosin-ATPase durch Aktivatoren und die resultierenden Aktivierungseffeke, *Z. Naturforsch.*, 76, 163, 1952.
6. Trentham, D. R., Eccleston, J. F., and Bagshaw, C. R., Kinetic analysis of ATPase mechanisms, *Q. Rev. Biophys.*, 9, 217, 1976.
7. Taylor, E. W., Mechanism of actomyosin ATPase and the problem of muscle contraction, *CRC Crit. Rev. Biochem.*, 6, 103, 1979.
8. Adelstein, R. S. and Eisenberg, E., Regulation and kinetics of the actin-myosin-ATP interaction, *Annu. Rev. Biochem.*, 49, 293, 1980.
9. Heilbrunn, L. V. and Wiercinsky, F. J., The action of various cations on muscle protoplasm, *J. Cell. Comp. Physiol.*, 29, 15, 1947.
10. Ebashi, S. and Ebashi, F., A new protein component participating in the superprecipitation of myosin B, *J. Biochem (Tokyo)*, 55, 604, 1964.
11. Ebashi, S. and Kodama, A., A new protein factor promoting aggregation of tropomyosin, *J. Biochem. (Tokyo)*, 58, 107, 1965.
12. Ebashi, S. and Kodama, A., Native tropomyosin-like action of troponin on trypsin treated myosin B, *J. Biochem (Tokyo)*, 60, 733, 1966.
13. Ebashi, S. and Nonomura, Y., Regulatory structural proteins of muscle, *Abstr. 7th Int. Congr. Biochem. Symp. VI*, 1967, 329.
14. Ebashi S. and Endo, M., Calcium ion and muscle contraction, *Prog. Biophys. Mol. Biol.*, 18, 123, 1968.
15. Hanson, J. and Lowy, J., The structure of actin, in *Biochemistry of Muscle Contraction*, Gergely, J., Ed., Little, Brown, Boston, 1962, 141.
16. Hanson, J. and Lowy, J., The structure of F-actin and of elements isolated from muscle, *J. Mol. Biol.*, 6, 46, 1963.
17. Kendrick-Jones, J., Lehman, W., and Szent-Gyorgyi, A. G., Regulation in molluscan muscle, *J. Mol. Biol.*, 54, 313, 1970.
18. Lehman, W., Kendrick-Jones, J., and Szent-Gyorgyi, A. G., Myosin-linked regulatory systems: comparative studies, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 319, 1972.

19. Lehman, W. and Szent-Gyorgyi, A. G., Distribution of actin control and myosin control in the animal kingdom, *J. Gen. Physiol.*, 66, 1, 1975.
20. Kendrick-Jones, J. and Scholey, J. M., Myosin-linked regulatory systems, *J. Muscle Res. Cell Motility*, 2, 347, 1981.
21. Cooke, R. and Stull, J. T., Myosin phosphorylation: a biochemical mechanism for regulating contractility, in *Cell and Muscle Motility*, Vol 1, Dowben, R. M. and Shay, J. W., Eds., Plenum Press, New York, 1981, 99.
22. Adelstein, R. S., Pato, M. D., Sellers, J. R., deLanerolle, P., and Conti, M. A., Regulation of contractile proteins by reversible phosphorylation of myosin and myosin kinase, in *Basic Biology of Muscles: A Comparative Approach*, Twarog, B. M., Levine, R. J. C., and Dewey, M. M., Eds., Raven Press, New York, 1982, 273.
23. Ebashi, S., Diversity of regulatory mechanisms and regulation in smooth muscle, in *Basic Biology of Muscles: A Comparative Approach*, Twarog, B. M., Levin, R. J. C., and Dewey, M. M., Eds., Raven Press, New York, 1982, 283.
24. Ebashi, S., Nonomura, Y., Nakamura, S., Nakasone, H., and Kohama, K., Regulatory mechanism in smooth muscle: actin-linked regulation, *Fed. Proc.*, 41, 2863, 1982.
25. Hartshorne, D. J. and Siemankowski, R., Regulation of smooth muscle actomyosin, *Annu. Rev. Physiol.*, 43, 519, 1981.
26. Barany, M. and Barany, K., Protein phosphorylation in cardiac and vascular smooth muscle, *Am. J. Physiol.*, 241, H117, 1981.
27. Perry, S. V., Cole, H. A., Grand, R. J. A., and Levine, B. A. Comparative aspects of the regulation of contraction in vertebrate muscle, in *Basic Biology of Muscles: A Comparative Approach*, Twarog, B. M., Levine, R. J. C., and Dewey, M. M., Eds., Raven Press, New York, 1982, 243.
28. Potter, J. D. and Johnson, J. D., Troponin, in *Calcium and Cell Function*, Vol. 2, Academic Press, New York, 1982, 145.
29. Perry, S. V., Structure and function relationships in troponin and calmodulin regulatory systems, in *Muscle Contraction: Its Regulatory Mechanisms*, Ebashi, S., Maruyama, K., and Endo, M., Eds., Japan Scientific Societies Press, Tokyo, 1980, 207.
30. Kretsinger, R. H., Calcium-binding proteins. *Annu. Rev. Biochem.*, 45, 239, 1976.
31. Kretsinger, R. H., Structure and evolution of calcium-modulated proteins, *CRC Crit. Rev. Biochem.*, 8, 119, 1980.
32. Levine, B. A. and Williams, R. J. P., Calcium binding to proteins and other large biological anion centers in *Calcium and Cell Function*, Vol. 2, Academic Press, New York, 1982, 1.
33. Seamon, K. B., Structural studies on high affinity calcium binding proteins: skeletal troponin-C and brain calmodulin, in *Biochemical Structure Determination by NMR*, Bothnerby, A. A., Glickson, J. D., and Sykes, B. D., Eds., Marcel Dekker, New York, 1982, 189.
34. Korn, E. D., Actin polymerization and its regulation by proteins from nonmuscle cells, *Physiol. Rev.*, 62, 672, 1982.
35. Craig, S. W. and Pollard, T. D., Actin-binding proteins, *Trends Biochem. Sci.*, 7, 88, 1982.
36. Smillie, L. B., Structure and functions of tropomyosins from muscle and non-muscle sources, *Trends Biochem. Sci.*, 4, 151, 1979.
37. Talbot, J. A. and Hodges, R. S., Tropomyosin: a model protein for studying coiled-coil and alpha-helix stabilization, *Acc. Chem. Res.*, 15, 224, 1982.
38. McCubbiin, W. D. and Kay, C. M., Calcium-induced conformational changes in the troponin-tropomyosin complexes of skeletal and cardiac muscle and their roles in the regulation of contraction-relaxation, *Acc. Chem. Res.*, 13, 185, 1980.
39. Perry, S. V., The regulation of contractile activity in muscle, *Biochem. Soc. Trans.*, 7, 21, 1979.
40. Murray, J. M., Weber, A., and Wegner, A., Tropomyosin and the various states of the regulated actin filament, in *Muscle Contraction: Its Regulatory Mechanisms*, Ebashi, S., Maruyama, K., and Endo, M., Eds., Japan Scientific Societies Press, Tokyo, 1980, 221.
41. Trueblood, C. E., Walsh, T. P., and Weber, A., Is the steric model of tropomyosin action valid?, in *Basic Biology of Muscles: A Comparative Approach*, Twarog, B. M., Levine, R. J. C., and Dewey, M. M., Eds., Raven Press, New York, 1982, 223.
42. Squire, J., Muscle regulation: a decade of the steric blocking model, *Nature (London)*, 291, 614, 1981.
43. Chantler, P., Retreats from the steric blocking of muscle contraction, *Nature (London)*, 298, 120, 1982.
44. Eisenberg, E. and Greene, L., The relation of muscle biochemistry to muscle physiology, *Annu. Rev. Physiol.*, 42, 293, 1980.
45. Drabikowski, W., Barylko, B., Dabrowska, R., and Nowak, E., Binding of calcium by troponin, *Bull. Acad. Pol. Sci. Ser. Sci. Biol.*, 16, 397, 1968.
46. Ebashi, S., Kodama, A., and Ebashi, F., Troponin I: preparation and physiological function, *J. Biochem. (Tokyo)*, 64, 465, 1968.

47. Yasui, B., Fuchs, F., and Briggs, F. N., The role of the sulfhydryl groups of tropomyosin and troponin in the calcium control of actomyosin contractility, *J. Biol. Chem.*, 243, 735, 1968.
48. Fuchs, F. and Briggs, F. N., The site of calcium bonding in relation to the activation of myofibrillar contraction, *J. Gen. Physiol.*, 51, 655, 1968.
49. Ebashi, S., Endo, M., and Ohtsuki, I., Control of muscle contraction *Q. Rev. Biophys.*, 2, 351, 1969.
50. Wakabayashi, T. and Ebashi, S., Reversible change in the physical state of troponin induced by calcium ion, *J. Biochem. (Tokyo)*, 64, 731, 1968.
51. Arai, K. I. and Watanabe, S., A study of troponin, a myofibrillar protein from rabbit skeletal muscle, *J. Biol. Chem.*, 243, 5670, 1968.
52. Schaub, M. C. and Perry, S. V., The relaxing protein system of striated muscle. Resolution of troponin complex into inhibitory and calcium ion sensitizing factors and their relationship to tropomyosin, *Biochem. J.*, 115, 993, 1969.
53. Hartshorne, D. J. and Mueller, H., Fractionation of troponin into two distinct proteins, *Biochem. Biophys. Res. Commun.*, 31, 647, 1968.
54. Schaub, M. C., Perry, S. V., and Hackcer, W., The regulatory proteins of the myofibril. Characterization and biological activity of the calcium-sensitizing factor (Troponin A), *Biochem. J.*, 126, 237, 1972.
55. Hartshorne, D. J. and Pyun, H. Y., Calcium binding by the troponin complex, the purification properties of troponin A, *Biochim. Biophys. Acta*, 229, 698, 1971.
56. Wilkinson, J. M., Perry, S. V., Cole, H. A., and Trayer, I. P., The regulatory proteins of the myofibril. Separation and biological activity of the components of inhibitory-factor preparations, *Biochem. J.*, 127, 215, 1972.
57. Drabikowski, W., Rafalowska, U., Dabrowska, R., Szpacenko, A., and Barylko, B., The effect of proteolytic enzymes on the troponin complex, *Fed. Eur. Biochem. Soc. Lett.*, 19, 259, 1971.
58. Ebashi, S., Wakabayashi, T., and Ebashi, F., Troponin and its components, *J. Biochem. (Tokyo)*, 69, 441, 1971.
59. Greaser, M. L. and Gergely, J., Reconstitution of troponin activity from three protein components, *J. Biol. Chem.*, 246, 4226, 1971.
60. Greaser, M. L. and Gergely, J., Purification and properties of the components from troponin, *J. Biol. Chem.*, 248, 2125, 1973.
61. Ebashi, S., Ohtsuki, I., and Mihashi, K., Regulatory proteins of muscle with special reference to troponin, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 215, 1972.
62. Eisenberg, E. and Kielley, W. W., Evidence for a refractory state of heavy meromyosin and subfragment-1 unable to bind to actin in the presence of ATP, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 145, 1972.
63. Greaser, M. L., Yamaguchi, M., Brekke, C., and Gergely, J., Troponin subunits and their interactions, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 235, 1972.
64. Potter, J. D., The content of troponin, tropomyosin, actin and myosin in rabbit skeletal muscle myofibrils, *Arch. Biochem. Biophys.*, 162, 436, 1974.
65. Sperling, J. E., Feldmann, K., Meyer, H., Jahnke, U., and Heilmeyer, L. M. G., Jr., Isolation, characterization and phosphorylation pattern of the troponin complexes TI2C and I2C, *Eur. J. Biochem.*, 101, 581, 1979.
66. Hartshorne, D. J. and Driezen, P., Studies on the subunit composition of troponin, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 225, 1972.
67. Wilkinson, J. M., Perry, S. V., Cole, H. A., and Trayer, I. P., The regulatory proteins of the myofibril. Separation and biological activity of the components of inhibitory-factor preparations, *Biochem. J.*, 127, 215, 1972.
68. Margossian, S. S. and Stafford, W. F., III, Calcium-induced dimerization of troponin C, *J. Biol. Chem.*, 257, 1160, 1982.
69. Fuchs, F., A rapid ultrafiltration procedure for the estimation of calcium-protein binding constants, *Int. J. Pept. Protein Res.*, 4, 147, 1972.
70. Drabikowski, W. and Barylko, B., Calcium binding by troponin, *Acta Biochim. Pol.*, 18, 353, 1971.
71. Klotz, I. M., Protein interactions with small molecules, *Acct. Chem. Res.*, 7, 162, 1974.
72. Bremel, R. D. and Weber, A., Cooperation within actin filament in vertebrate skeletal muscle, *Nature New Biol.*, 238, 97, 1972.
73. Potter, J. D. and Gergely, J., The calcium and magnesium binding sites on troponin and their role in the regulation of myofibrillar adenosine triphosphatase, *J. Biol. Chem.*, 250, 4628, 1975.
74. Ikemoto, N., The calcium binding sites involved in the regulation of the purified adenosine triphosphatase of the sarcoplasmic reticulum, *J. Biol. Chem.*, 249, 649, 1974.
75. Ikemoto, N., Nagy, B., Bhatnagar, G. M., and Gergely, J., Studies on a metal-binding protein of the sarcoplasmic reticulum, *J. Biol. Chem.*, 249, 2357, 1974.
76. Potter, J. D. and Johnson, J. D., The effect of magnesium on calcium binding to the calcium-specific sites of troponin, *Biophys. J.*, 25, 249a, 1979.

77. Collins, J. H., Potter, J. D., Horn, M. J., Wilshire, G., and Jackman, N., The amino acid sequence of rabbit skeletal muscle troponin C; gene replication and homology with calcium-binding proteins from carp and hake muscle, *FEBS Lett.*, 36, 268, 1973.
78. Collins, J. H., Greaser, M. L., Potter, J. D., and Horn, M. J., Determination of the amino acid sequence of troponin C from rabbit skeletal muscle, *J. Biol. Chem.*, 242, 6356, 1977.
79. Collins, J. H., Structure and evolution of troponin C and related proteins, *Soc. Exp. Biol. Symp.*, 30, 303, 1976.
80. Deuticke, H. J., Concerning the sedimentation constant of muscle proteins, *Phys. Chem.*, 224, 216, 1934.
81. Hamoir, G. and Konosu, S., Carp myogens of white and red muscles. General composition and isolation of low-molecular-weight components of abnormal amino acid composition, *Biochem. J.*, 96, 85, 1965.
82. Hamoir, G., The comparative biochemistry of fish sarcoplasmic proteins, *Acta Zool. Pathol. Antverp.*, 46, 69, 1968.
83. Konosu, S., Hamoir, G., and Pechere, J.-F., Carp myogens of white and red muscle. Properties and amino acid composition of the main low-molecular-weight components of white muscle, *Biochem. J.*, 96, 98, 1965.
84. Pechere, J.-F. and Focant, B., Carp myogens of white and red muscles. Gross isolation on sephadex columns of the low-molecular-weight components and examination of their participation in anaerobic glycogenolysis, *Biochem. J.*, 96, 113, 1965.
85. Pechere, J.-F. and Capony, J.-P., A comparison at the peptide level of muscular paravalbumins from several lower vertebrates, *Comp. Biochem. Physiol.*, 28, 1089, 1969.
86. Pechere, J.-F., Demaille, J., and Capony, J.-P., Muscular parvalbumins: preparative and analytical methods of general applicability, *Biochim. Biophys. Acta*, 236, 391, 1971.
87. Pechere, J.-F., Capony, J.-P., and Ryden, L., The primary structure of the major paravalbumin from hake muscle, *Eur. J. Biochem.*, 23, 421, 1971.
88. Benzonana, G., Capony, J.-P., and Pechere, J.-F., The binding of calcium to muscular parvalbumins, *Biochim. Biophys. Acta*, 278, 110, 1972.
89. Nockolds, C. E., Kretsinger, R. H., Coffee, C. J., and Bradshaw, R. A., Structure of a calcium-binding carp myogen, *Proc. Natl. Acad. Sci. U.S.A.*, 69, 581, 1972.
90. Pechere, J.-F., Capony, J.-P., Ryden, L., and Demaille, J., The amino acid sequence of the major parvalbumin from hake muscle, *Biochem. Biophys. Res. Cummun.*, 43, 1106, 1971.
91. Coffee, C. J. and Bradshaw, R. A., Carp muscle calcium-binding protein. I. Characterization of the tryptic peptides and the complete amino acid sequence of component B, *J. Biol. Chem.*, 248, 3305, 1973.
92. Kretsinger, R. H. and Nockolds, C. E., Carp muscle calcium-binding protein. II. Structure determination and general description, *J. Biol. Chem.*, 248, 3313, 1973.
93. Hendrickson, W. A. and Karle, J., Carp muscle calcium-binding protein. III. Phase refinement using the tangent formula, *J. Biol. Chem.*, 248, 3327, 1973.
94. Kretsinger, R. H. and Barry, C. D., The predicted structure of the calcium-binding component of troponin, *Biochim. Biophys. Acta*, 405, 40, 1975.
95. Mercola, D., Bullard, B., and Priest, J., Crystallization of troponin C, *Nature, (London)*, 254, 634, 1975.
96. Strasburg, G. M., Greaser, M. L., and Sundaralingam, M., X-ray diffraction studies of troponin C crystals from rabbit and chicken skeletal muscles, *J. Biol. Chem.*, 255, 3806, 1980.
96a. Gergely, J. and Leavis, P. C., The structure of troponin C and thin filament regulation in striated muscle, in *Muscle Contraction: Its Regulatory Mechanisms*, Ebashi, S., Maruyama, K., and Endo, M., Eds., Springer-Verlag, Berlin, 1980, 191.
97. Williams, R. J. P., Calcium chemistry and its relation to protein binding, in *Calcium Binding Proteins and Calcium Function*, Wasserman, R. H., Corradino, R. A., Carafoli, E., Kretsinger, R. H., MacLennan, D. H., and Siegel, F. L., Eds., North-Holland, New York, 1978, 3.
98. Cummings, S., Enderby, J. E., and Howe, R. A., Ion hydration in aqueous $CaCl_2$ solution, *J. Phys. C*, 13, 1, 1980.
99. Grabarek, Z. and Kuznicki, J., Homologous calcium binding proteins, *Postepy Biochem.*, 26, 209, 1980.
100. Weeds, A. G. and McLachlan, A. D., Structral homology of myosin alkali light chains, troponin C and carp calcium binding protein, *Nature (London)*, 252, 646, 1974.
101. Vogt, H. P., Strassburger, W., Wollmer, A., Fleischhaur, J., Bullard, B., and Mercola, D., Calcium binding by troponin-C and homologs is correlated with the position and linear density of "beta-turn forming" residues, *J. Theor. Biol.*, 7, 297, 1979.
102. Chou, P. Y. and Fasman, G. D., Conformational parameters for amino acids in helical, beta-sheet and random coil regions calculated from proteins, *Biochemistry*, 13, 211, 1974.

103. Potter, J. D., Johnson, J. D., Dedman, J. R., Schreiber, W. E., Mandel, F., Jackson, R. L., and Means, A. R. Calcium-binding proteins: relationship of binding, structure, conformation and biological function, in *Calcium Binding Proteins and calcium Function*, Wasserman, R. H., Corradino, R. A., Carafoli, E., Kritsinger, R. H., MacLennan, D. H., and Siegel, F. L., Eds., North-Holland, New York, 1977, 239.
104. Kretsinger, R. H., Calcium binding proteins and natural membranes, in *Perspectives in Membrane Biology*, Estrada, S. and Gitler, C., Eds., Academic Press, New York, 1974, 229.
105. Reid, R. E. and Hodges, R. S., Co-operativity and calcium/magnesium binding to troponin C and muscle calcium binding parvalbumin: an hypothesis, *J. Theor. Biol.*, 84, 401, 1980.
106. Reid, R. E., Clare, D. M., and Hodges, R. S., Synthetic analog of a high affinity calcium binding site in rabbit skeletal troponin C, *J. Biol. Chem.*, 255, 3642, 1980.
107. Reid, R. E., Gariepy, J., Saund, A. K., and Hodges, R. S., Calcium-induced protein folding, *J. Biol. Chem.*, 256, 2742,1981.
108. Coffee, C. J. and Solano, C., Preparation and properties of carp muscle parvalbumin fragments A (residues 1 to 750) and B (residues 76 to 108), *Biochim. Biophys. Acta*, 453, 67, 1976.
109. Leavis, P. C., Rosenfeld, S. S., Gergely, J., Grabarek, Z., and Drabikowski, W., Proteolytic fragments of troponin C., *J. Biol. Chem.*, 253, 5452, 1978.
110. Head, J. F. and Perry, S. V., The interaction of the calcium binding protein (troponin C) with bivalent cations and the inhibitory protein (troponin I), *Biochem. J.*, 137, 145, 1974.
111. Nagy, B. and Gergely, J., Extent and localization of conformational changes in troponin C caused by calcium binding, *J. Biol. Chem.*, 254, 12732, 1979.
112. McCubbin, W. D., Hincke, M. T., and Kay, C. M., The utility of the nitrotyrosine chromophore as a spectroscopic probe in troponin C and modulator protein, *Can. J. Biochem.*, 557, 15, 1979.
113. van Eerd, J.-P. and Kawasaki, Y.,Ca^{2+} induced conformational changes in the Ca^{2+} binding component of troponin, *Biochem. Biophys. Res. Commun.*, 47, 859, 1972.
114. Kawasaki, Y. and van Eerd, J.-P., The effects of Mg^{2+} on the conformation of the Ca^{2+}-binding component of troponin, *Biochem. Biophys. Res. Commun.*, 49, 898, 1972.
115. Leavis, P. L. and Lehrer, S. S., Intrinsic fluorescence studies on troponin C, *Arch. Biochem. Biophys.*, 187, 243, 1978.
116. Permyakov, E. A., Yarmolenko, V. V., and Korystova, A. A., An evaluation of the Ca^{2+} binding constants of troponin-C by means of intrinsic protein fluorescence, *Stud. Biophys.*, 83, 63, 1981.
117. Carew, E. B., Leavis, P. C., Stanley, H. E., and Gergely, J., A laser Raman spectroscopic study of Ca^{2+} binding to troponin C, *Biophys. J.*, 30, 351, 980.
118. Murray, A. C. and Kay, C. M., Hydrodynamic and optical properties of troponin A, Demonstration of conformational change upon binding calcium ion, *Biochemistry*, 11, 2622, 1972.
119. Johnson, J. D. and Potter, J. D., Detection of two classes of Ca^{2+} binding sites in troponin C with circular dichroism and tyrosine fluorescence, *J. Biol. Chem.*, 253, 3775, 1978.
120. Hincke, M. T., McCubbin, W. D., and Kay, C. M., Calcium binding properties of cardiac and skeletal troponin C as determined by circular dichroism and ultraviolet difference spectroscopy, *Can. J. Biochem.*, 56, 384, 1978.
121. Leavis, P. C., Nagy, B., Lehrer, S. S., Bialkowska, H., and Gergely, J., Terbium binding to troponin C: binding stoichiometry and structural changes induced in the protein, *Arch. Biochem. Biophys.*, 200, 17, 1980.
122. McCubbin, N. D. and Kay, C. M., Physicochemical and biological studies on the metal induced conformational change in troponin A. Implication of carboxyl groups in the binding of calcium ion, *Biochemistry*, 12, 4228, 1973.
123. Gruda, J., Therien, H.-M.,and Cermakian, E., The effects of calcium and magnesium ions on the secondary structure of calcium binding component of troponin, *Biochem. Biophys. Res. Commun.* 52, 1307, 1973.
124. Byers, D. M. and Kay, C. M., Hydrodynamic properties of bovine cardiac troponin C, *Biochemistry*, 21, 229, 1982.
125. Grabarek, Z., Drabikowski, W., Vinokurov, L., and Lu, R. C., Digestion of troponin C with trypsin in the presence and absence of Ca^{2+}. Identification of cleavage points, *Biochim. Biophys. Acta*, 671, 227, 1981.
126. Levine, B. A., Moore, G. R., Ratcliffe, R. G., and Williams, R. J. P., Nuclear magnetic resonance studies of the solution structure of proteins, in *International Review of Biochemistry*, Offord, R.E., Ed., University Park Press, Baltimore, 24, 77, 1979.
127. Levine, B. A., Mercola, D., Coffman, D., and Thornton, J. M., Calcium binding by troponin C. A proton magnetic resonance study, *J. Mol. Biol.*, 115, 743, 1977.
128. Seamon, K. B., Hartshorne, D. J., and Bothner-By, A., Ca^{2+} and Mg^{2+}-dependent conformations of troponin C as determined by ^{1}H and ^{19}F nuclear magnetic resonance, *Biochemistry*, 16, 4039, 1977.
129. Levine, B. A., Mercola, D., and Thornton, J. M., Proton magnetic resonance study of troponin C, *FEBS Lett.*, 61, 218, 1976.

130. Levine, B. A., Thornton, J. M., Fernandes, R., Kelly, C. M., and Mercola D., Comparison of the calcium- and magnesium-induced structural changes of troponin-C. A proton magnetic resonance study, *Biochim. Biophys. Acta*, 535, 11, 1978.
131. Hincke, M. T., Sykes, B. D., and Kay, C. M., Hydrogen-1 nuclear magnetic resonance investigation of bovine cardiac troponin C. Comparison of tyrosyl assignments and calcium-induced structural changes to those of two homologous proteins, rabbit skeletal troponin C and bovine brain calmodulin, *Biochemistry*, 20, 3286, 1981.
132. Leavis, P. L., Gergely, J., Grabarek, Z., Drabikowski, W., and Levine, B. A., Calcium and the conformational states of troponin C, in *Calcium Binding Proteins: Structure and Function*, Siegel, F. L., Carafoli, E., Kretsinger, R. H., MacLennan, D. H., and Wasserman, R. H., Eds., North-Holland, New York, 1980, 271.
133. Leavis, P. C., Grabarek, Z., Gregley, J., and Levine, B. A., An ^{1}H-NMR study of the structure and interactions of the high affinity regions of troponin C, *Biophys. J.*, 37, 35a, 1982.
134. Potter, J. D., Seidel, J. C., Leavis, P. C., Lehrer, S. S., and Gergely, J., Effects of Ca^{2+} binding on troponin C, *J. Biol. Chem.*, 251, 7551, 1976.
135. Iio, T. and Kondo, H., A fluorescence stopped-flow study on troponin labeled with N-ethyl maleimide and N-(p-(2-benzimidazolyl)phenyl) maleimide, *J. Biochem. (Tokyo)*, 86, 1883, 1979.
136. Iio, T. and Kondo, H., Fluorescence stopped-flow study of N-(-7-dimethylamido-4-methyl-3-coumarinyl)-maleimide-labeled troponin-C, *J. Biochem. (Tokyo)*, 88, 1087, 1980.
137. Iio, T. and Kondo, H., Fluorescence titration and fluorescence stopped-flow studies on skeletal troponin C labeled with fluorescence maleimide reagent or dansylaziridine, *J. Biochem. (Tokyo)*, 90, 163, 1981.
138. Ebashi, S., Ohnishi, S., Abe, S., and Maruyama, K., A spin-label study on calcium-induced conformational changes of troponin components, *J. Biochem. (Tokyo)*, 75, 211, 1974.
139. Johnson, J. D., Collins, J. H., Robertson, S. P., and Potter, J. D., A fluorescent probe study of Ca^{2+} binding to the Ca^{2+}-specific sties of cardiac troponin and troponin C, *J. Biol. Chem.*, 255, 9635, 1980.
140. Dobrovol'skii, A. B. and Gusev, N. B., Role of the Ca^{2+}-specific binding sites in the troponin structure, *Biokhimiya*, 43, 1695, 1978.
141. Hitchcock, S. E., Study of the structure of troponin-C by measuring the relative reactivities of lysines with acetic anhydride, *J. Mol. Biol.*, 147, 1553, 1981.
142. Evans, J. S., Levine, B. A., Leavis, P. L., Gergely, J., Grabarek, Z., and Drabikowski, W., Proton magnetic resonance studies on proteolytic fragments of troponin-C. Structural homology with the native molecule, *Biochim. Biophys. Acta*, 623, 10, 1980.
143. Leavis, P. L. and Kraft, E. L., Calcium binding to cardiac troponin C., *Arch. Biochem. Biophys.*, 186, 411, 1978.
144. Holroyde, J. M., Robertson, S. P., Johnson, J. D., Solaro, R. J., and Potter, J. D., The calcium and magnesium binding sites on cardiac troponin and their role in the regulation of myofibrillar adenosine triphosphatase, *J. Biol. Chem.*, 255, 11688, 1980.
145. van Eerd, J. P. and Takahashi, K., The amino acid sequence of bovine cardiac troponin-C. Comparison with rabbit skeletal troponin-C, *Biochem. Biophys. Res. Commun.*, 64, 122, 1975.
146. Wilkinson, J. M., Troponin C from rabbit slow skeletal and cardiac muscle is the product of a single gene, *Eur. J. Biochem.*, 103, 179, 1980.
147. Sin, I. L., Fernandes, R., and Mercola, D., Direct identification of the high and low affinity calcium binding sites of troponin C, *Biochem. Biophys. Res. Commun.*, 82, 1132, 1978.
148. Cheung, H. C., Conformational dynamics of fluorescence-labeled troponin-C, in *Calcium Binding Proteins and Calcium Function*, Wasserman, R. H., Coradino, R. A., Carafoli, E., Kretsinger, R. H., MacLennan, D. H., and Siegel, F. L., Eds., North-Holland, New York, 1977, 263.
149. Iio, T., Mihashi, K., and Kondo, H., Kinetics of conformational change of troponin C induced by calcium, *J. Biochem, (Tokyo)*, 79, 689, 1976.
150. Iio, T., Mihashi, K., and Kondo, H., Kinetics of conformational change of troponin-C induced by binding or removal of calcium ion, *J. Biochem. (Tokyo)*, 83, 961, 1978.
151. Johnson, J. D., Collins, J. H., and Potter, J. D., Dansylaziridine-labeled troponin C. A fluorescent probe of Ca^{2+} binding to the Ca^{2+}-specific regulatory sites, *J. Biol Chem.*, 253, 6451, 1978.
152. Johnson, J. D., Charlton, S. C., and Potter, J. D., A fluorescence stopped-flow analysis of Ca^{2+} exchange with troponin C, *J. Biol. Chem.*, 254, 3497, 1979.
153. Andersson, T., Drakenberg, T., Forsen, S., and Thulin, E., A ^{43}CaNMR and ^{25}MgNMR study of rabbit skeletal muscle troponin C, *FEBS Lett.*, 125, 39, 1981.
154. Robertson, S. P., Johnson, J. D., and Potter, J. D., Time course of Ca^{2+} exchange with calmodulin, troponin, parvalbumin and myosin in response to transient increases in Ca^{2+}, *Biophys. J.*, 34, 559, 1981.

155. Fuchs, F., Ion exchange properties of the calcium receptor site of troponin, *Biochim. Biophys. Acta*, 245, 221, 1971.
156. Edwards, C., Lorkovic, H., and Weber, A., The effect of the replacement of calcium by strontium on excitation-contraction coupling in frog skeletal muscle, *J. Physiol. (London)*, 186, 295, 1968.
157. Lehrer, S. S. and Leavis, P. C., Fluorescence and conformational changes caused by proton binding to troponin C, *Biochem. Biophys. Res. Commun.*, 58, 159, 1974.
158. Robertson, S. P., Johnson, J. D., and Potter, J. D., The effect of pH on calcium binding to the Ca^{2+}-Mg^{2+} and the Ca^{2+}-specific sites of rabbit skeletal TnC, *Biophys. J.*, 21, 16a, 1978.
159. Delville, A., Grandjean, J., Laszlo, P., Gerday, C., Grabarek, Z., and Drabikowski, W., Sodium-23 nuclear magnetic resonance as an indicator of sodium binding to troponin C and tryptic fragments in relation to calcium content and attendant conformational changes, *Eur. J. Biochem.*, 105, 289, 1980.
160. Martin, R. B. and Richardson, F. S., Lanthanides as probes for calcium in biological systems, *Q. Rev. Biophys.*, 12, 181, 1979.
161. Moews, P. C. and Kretsinger, R. H., Terbium replacement of calcium in carp muscle calcium-binding parvalbumin: an X-ray crystallographic study, *J. Mol. Biol.*, 91, 229, 1975.
162. Nelson, D. J., Miller, T. L., and Martin, R. B., Non-cooperative Ca(II) removal and terbium(III) substitution in carp muscle calcium binding parvalbumin, *Bioinorg. Chem.*, 7, 325, 1977.
163. Sowadski, J., Cornick, G., and Kretsinger, R. H., Terbium replacement of calcium in parvalbumin, *J. Mol. Biol.*, 124, 123, 1978.
164. Miller, T. L., Nelson, D. J., Britain, H. G., Richardson, F. S., Martin, R. B., and Kay, C. M., Calcium binding sites of rabbit troponin and carp parvalbumin, *FEBS Lett.*, 58, 262, 1975.
165. Britain, H. G., Richardson, F. S., Martin, R. F., Burtnick, L. D., and Kay, C. M., Circularly polarized emission of terbium(III) substituted bovine cardiac troponin-C, *Biochem. Biophys. Res. Commun.*, 68, 1013, 1976.
166. Wang, C.-L. A., Leavis, P. C., Horrocks, W., DeW., Jr., and Gergely, J., Binding of lanthanide ions to troponin C, *Biochemistry*, 20, 2439, 1981.
167. Oikawa, K., McCubbin, W. D., and Kay, C. M., The effects of terbium and lanthanum on the biological activity of some representative muscle protein systems, *FEBS Lett.*, 118, 137, 1980.
168. Weeks, R. A. and Perry, S. V. Characterization of a region of the primary sequence of troponin C involved in calcium ion-dependent interaction with troponin I, *Biochem. J.*, 173, 449, 1978.
169. Grabarek, Z., Drabikowski, W., Leavis, P. L., Rosenfeld, S. S., and Gergely, J., Proteolytic fragments of troponin C. Interactions with the other troponin subunits and biological activity, *J. Biol. Chem.*, 256, 13121, 1981.
170. Ohara, O., Takahashi, S., and Ooi, T., A study on troponin-T and troponin-C interaction by cross-linking reagents, in *Muscle Contraction: Its Regulatory Mechanisms*, Ebashi, S., Maruyama, K., and Endo, M., Eds., Japan Scientific Societies Press, Tokyo, 1980, 259.
171. Perry, S. V., Cole, H. A., Head, J. F., and Wilson, F. J., Localization and mode of action of the inhibitory protein component of the troponin complex, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 251, 1972.
172. Potter, J. D. and Gergely, J., Troponin, tropomyosin and actin interactions in the calcium regulation of muscle contraction, *Biochemistry*, 13, 2697, 1974.
173. Castellani, L., Morris, E. P., and O'Brien, E. J., Calmodulin as a model for troponin-C, *Biochem. Biophys. Res. Commun.*, 96, 558, 1980.
174. Wilkinson, J. M. and Grand, R. J. A., The amino acid sequence of tropinin I from rabbit skeletal muscle, *Biochem. J.*, 149, 493, 1975.
175. Wilkinson, J. M., The preparation and properties of the components of troponin, *Biochim. Biophys. Acta*, 359, 379, 1974.
176. Horwitz, J., Bullard, B., and Mercola, D., Interaction of troponin subunits. The interaction between the inhibitory and tropomyosin binding subunits, *J. Biol. Chem.*, 254, 350, 1979.
177. Hincke, M. T., McCubbin, W. D., and Kay, C. M., The interaction between beef cardiac troponin T and troponin I as demonstrated by ultraviolet absorption difference spectroscopy, circular dichroism and gel filtration, *Can. J. Biochem.*, 57, 768, 1979.
178. Syska, H., Wilkinson, J. M., Grand, R. J. A., and Perry, S. V., The relationship between biological activity and primary structure of troponin I from white skeletal muscle of the rabbit, *Biochem. J.*, 153, 375, 1976.
179. Moir, A. J. G., Wilkinson, J. M., and Perry, S. V., The phosphorylation sites of troponin I from white skeletal muscle of the rabbit, *FEBS Lett.*, 42, 253, 1974.
180. Huang, T. S., Bylund, D. B., Stull, J. T., and Krebs, E. G., The amino acid sequences of the phosphorylated sites in troponin I from rabbit skeletal muscle, *FEBS Lett.*, 42, 249, 1974.
181. Perry, S. V. and Cole, H. A., Phosphorylation of troponin and the effects of interactions between the components of the complex, *Biochem. J.*, 141, 733, 1974.

182. Perry, S. V., Cole, H. A., Morgan, M., Moir, A. J. G., and Pires, E., Phosphorylation of the proteins of the myofibril, *Proc. 9th FEBS Meet.*, 31, 163, 1974.
183. Grand, R. J. A., Wilkinson, J. M., and Mole, L. E., The amino acid sequence of rabbit cardiac troponin I, *Biochem. J.*, 159, 633, 1976.
184. Moir, A. J. G. and Perry, S. V., The sites of phosphorylation of rabbit cardiac troponin I by adenosine 3′,5′-cyclic monophosphate-dependent protein kinase, *Biochem. J.*, 167, 33, 1977.
185. Moir, A. J. G. and Perry, S. V., Phosphorylation of rabbit cardiac muscle troponin I by phosphorylase kinase, *Biochem. J.*, 191, 547, 1980.
186. England, P. J., Correlation between contraction and phosphorylation of the inhibitory subunit of troponin in perfused rat heart, *FEBS Lett.*, 50, 57, 1975.
187. Solaro, R. J., Moir, A. J. G., and Perry, S. V., Phosphorylation of troponin I and the inotropic effect of adrenaline on perfused rabbit heart, *Nature (London)*, 262, 615, 1976.
188. Moir, A. J. G., Solaro, R. J., and Perry, S. V., The site of the phosphorylation of troponin I in the perfused rabbit heart: the effect of adrenaline, *Biochem. J.*, 185, 505, 1980.
189. Wilkinson, J. M. and Grand, R. J. A., The amino acid sequence of troponin I from rabbit white skeletal muscle, *Proc. 9th FEBS Meet.*, 31, 137, 1974.
190. Hartshorne, D. J., Theiner, M., and Mueller, H., Studies on troponin, *Biochim. Biophys. Acta*, 175, 320, 1969.
191. Wu, C.-S. C. and Yang, J. T., Re-examination of the conformation of muscle proteins by optical activity, *Biochemistry*, 15, 3007, 1976.
192. Lu, R. C., Gowell, E., Wong, A., and Leavis, P. C., The interaction of troponin I with troponin C studied by surface labeling, *Biophys. J.*, 33, 146a, 1981.
193. Hitchcock-Degregori, S. E., Study of the structure of troponin I by measuring the relative reactivity of lysines with acetic anhydride, *Biophys J.*, 36, 237a, 1981.
194. Talbot, J. A. and Hodges, R. S., Synthetic studies on the inhibitory region of rabbit skeletal troponin I, *J. Biol. Chem.*, 256, 2798, 1981.
195. Nozaki, S., Kobayashi, K., Katayama, E., and Muramatsu, I., Synthetic studies on troponin I active site. Preparation of a pentadecapeptide with inhibitory activity toward actomyosin triphosphatase, *Chem. Lett.*, 3, 345, 1980.
196. Grand, R. J. A., Levine, B. A., and Perry, S. V., Proton-magnetic-resonance studies on the interaction of rabbit-skeletal muscle troponin I with troponin C and actin, *Biochem. J.*, 203, 61, 1982.
197. Pearlstone, J. R. and Smillie, L. B., Amino acid sequence of tropomyosin-binding component of rabbit skeletal muscle troponin, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 1902, 1976.
198. Pearlstone, J. R. and Smillie, L. B., Troponin T fragments: physical properties and binding to troponin C, *Can. J. Biochem.*, 56, 521, 1978.
199. Prendergast, F. G. and Potter, J. D., Solution conformation and hydrodynamic properties of rabbit skeletal troponin T, *Biophys. J.*, 25, 250a, 1979.
200. Ohtsuki, I., Molecular arrangement of troponin T in the thin filament, *J. Biochem. (Tokyo)*, 86, 491, 1979.
200a. Flicker, P. F., Phillips, G. N., Jr., and Cohen, C., Troponin and its interactions with tropomyosin, *J. Mol. Biol.*, 162, 495, 1982.
201. Moir, A. J. G., Cole, H. A., and Perry, S. V., The phosophrylation sites of troponin T from white skeletal muscle and the effects of interaction with troponin C on their phosphorylation by phosphorylase kinase, *Biochem. J.*, 161, 371, 1977.
202. Risnik, V. V., Dobrovolskii, A. B., Gusev, N. B., and Severin, S. E., Phosphorylase kinase phosphorylation of skeletal muscle troponin T, *Biochem. J.*, 191, 851, 1980.
203. Ebashi, S., Ohtsuki, I., and Mihashi, K., Regulatory proteins of muscle with special reference to troponin, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 215, 1973.
204. Margossian, S. S. and Cohen, C., Troponin subunit interactions, *J. Mol. Biol.*, 81, 409, 1973.
205. Hartshorne, D. J. and Mueller, H., The preparation of tropomyosin and troponin from natural actomyosin, *Biochim. Biophys. Acta*, 175, 301, 1969.
206. Hitchcock, S. E., Cross-linking of troponin with dimethylimido esters, *Biochemistry*, 14, 5162, 1975.
207. Sutoh, K., Direct evidence for the calcium-induced change in the quaternary structure of troponin in situ. Millisecond cross-linking of troponin components by a photosensitive heterobifunctional reagent, *Biochemistry*, 19, 1977, 1980.
208. Pearlstone, J. R. and Smillie, L. B., Identification of a second binding region on rabbit skeletal troponin T for alpha-tropomyosin, *FEBS Lett.*, 128, 119, 1981.
209. Hitchcock, S. E., Zimmerman, C. J., and Smalley, C., Study of the structure of troponin T by measuring the relative reactivities of lysines with acetic anhydride, *J. Mol. Biol.*, 147, 125, 1981.
210. Pearlstone, J. R. and Smillie, L. B., The binding sites of rabbit skeletal troponin I on troponin T, *Can. J. Biochem.*, 58, 649, 1980.
211. Jackson, P., Amphlett, G. N., and Perry, S. V., The primary structure of troponin T and the interaction with tropomyosin, *Biochem. J.*, 151, 85, 1975.

212. Pato, M. D., Mak, A. S., and Smillie, L. B., Fragments of rabbit striated muscle alpha-tropomyosin. II. Binding to troponin T., *J. Biol. Chem.*, 256, 602, 1981.
213. Mak, A. S. and Smillie, L. B. Structural interpretation of the two-site binding of troponin on the muscle thin filament, *J. Mol. Biol.*, 149, 541, 1981.
214. Nagano, K., Miyamoto, S., Matsumura, M., and Ohtsuki, I., Possible formation of a triple-stranded coiled-coil region in tropomyosin-troponin T binding complex, *J. Mol. Biol.*, 141, 217, 1980.
215. Bailey, K., Tropomyosin: a new asymmetric protein component of the muscle fibril, *Biochem. J.*, 43, 271, 1948.
216. Ebashi, S., Third component participating in the superprecipitation of natural actomyosin, *Nature (London)*, 200, 1010, 1963.
217. Martonosi, A., Studies on actin. VII. Ultracentrifugal analysis of partially polymerized actin solutions, *J. Biol. Chem.*, 237, 2795, 1962.
218. Laki, K., Maruyama, K., and Kominz, D. R., Evidence for the interaction between tropomosin and actin, *Arch. Biochem. Biophys.*, 98, 323, 1962.
219. Moore, P. B., Huxley, H. E., and DeRosier, D. J., Three-dimensional reconstruction of F-actin, thin filaments and decorated thin filaments, *J. Mol. Biol.*, 50, 279, 1970.
220. Hanson, J., X-ray diffraction studies of muscle, *Q. Rev. Biophys.*, 1, 177, 1968.
221. Ebashi, S., Endo, M., and Ohtsuki, I., Control of muscle contraction, *Q.Rev. Biophys.*, 2, 351, 1969.
222. Woods, W. F., Molecular weight and sub-unit structure of tropomyosin B, *J. Biol. Chem.*, 242, 2859, 1967.
223. Cummins, P. and Perry, S. V., The subunits and biological activity of polymorphic forms of tropomyosin, *Biochem. J.*, 133, 765, 1973.
224. Yamaguchi, M., Greaser, M., and Cassens, R. G., Interaction of troponin subunits with different forms of tropomyosin, *J. Ultrastruct. Res.*, 33, 48, 1974.
225. Hodges, R. S., Sodek, J., Smillie, L. B., and Jurasek, L., Tropomyosin: amino acid sequence and coiled-coil structure, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 299, 1972.
226. Sodek, J., Hodges, R. S., Smillie, L. B., and Jusek, L., Amino-acid sequence of rabbit skeletal tropomyosin and its coiled-coil structure, *Proc. Natl. Acad. Sci. U.S.A.*, 69, 3800, 1972.
227. Mak, A. S., Smillie, L. B., and Stewart, G. R., A comparison of the amino acid sequences of rabbit skeletal muscle alpha- and beta-tropomyosins, *J. Biol. Chem.*, 255, 3647, 1980.
228. Cummins, P. B. and Perry, S. V., Chemical and immunochemical characteristics of tropomyosins from striated and smooth muscle, *Biochem. J.*, 141, 43, 1974.
229. Leger, J., Bouveret, P., Schwartz, K., and Swynghedauw, B., A comparative study of skeletal and cardiac tropomyosins. Subunits, thiol group content and biological activity, *Pfluegers Arch.*, 362, 271, 1976.
230. Dhoot, G. K. and Perry, S. V., Distribution of polymorphic forms of troponin components and tropomyosin in skeletal muscle, *Nature (London)*, 278, 714, 1979.
231. Roy, R. K., Sreter, F. A., and Sarkar, S. Tropomyosin subunits and myosin light chains of avian and mammalian muscle: differential gene expression during development, *Aging*, 6, 23, 1978.
232. Strasburg, G. M. and Greaser, M. L., The native subunit pattern of tropomyosin, *FEBS Lett.*, 72, 11, 1976.
233. Bronson, D. D. and Schachat, F. H., Heterogeneity of contractile proteins, *J. Biol. Chem.*, 257, 3937, 1982.
234. Amphlett, G. W., Syska, H., and Perry, S. V., The polymorphic forms of tropomyosin and troponin I in developing rabbit skeletal muscle, *FEBS Lett.*, 63, 22, 1976.
235. Monterras, D., Fiszman, M. Y., and Gros, F., Changes in tropomyosin during development of chick embryonic skeletal muscles in vivo and during differentiation of chick muscle cells in vitro, *J. Biol. Chem.*, 257, 545, 1982.
236. Holtzer, A., Clark, R., and Lowey, S., The conformation of native and denatured tropomyosin B, *Biochemistry*, 4, 2401, 1965.
237. Cohen, C. and Holmes, K. C., X-ray diffraction evidence for alpha-helical coiled-coils in native muscle, *J. Mol. Biol.*, 6, 423, 1963.
238. Parry, D. A. D., Analysis of the primary sequence of alpha-tropomyosin from rabbit skeletal muscle, *J. Mol. Biol.*, 98, 519, 1975.
239. Stone, D., Sodek, J., Johnson, P., and Smillie, L. B., Tropomyosin: correlation of amino acid sequence and structure, *Proc. 9th Fed. Eur. Biochem. Soc. Meet.*, 31, 125, 1974.
240. Hodges, R. S., Saund, A. K., Chong, P. C. S., St-Pierre, S. A., and Reid, R. E., Synthetic model for two-stranded alpha-helical coiled-coils, *J. Biol. Chem.*, 256, 1214, 1981.
241. McLachlan, A. D. and Stewart, M., Tropomyosin coiled-coil interactions: evidence for an unstaggered structure, *J. Mol. Biol.*, 98, 293, 1975.
242. Crick, F. H. C., The packing of alpha-helices; simple coiled-coils, *Acta Cryst.*, 6, 689, 1953.

243. Stewart, M., Tropomyosin: evidence for no stagger between chains, *FEBS Lett.*, 53, 5, 1975.
244. Johnson, P. and Smillie, L. B., Rabbit skeletal alpha-tropomyosin chains are in register, *Biochem. Biophys. Res. Commun.*, 64, 1316, 1975.
245. Lehrer, S. S., Intramolecular crosslinking of tropomyosin via disulfide bond formation: evidence for chain register, *Proc. Natl. Acad. Sci. U.S.A.*, 72, 3377, 1975.
246. Bullard, B., Mercola, D. A., and Mommaerts, W. F. H. M., The origin of the tyrosyl circular dichroism of tropomyosin, *Biochim. Biophys. Acta*, 434, 90, 1976.
246a. Nagy, B., Circular dichroic and perturbation spectra of aromatic chromophores in rabbit tropomyosin, *J. Biol. Chem.*, 252, 4557, 1977.
247. Betcher-Lange, S. L. and Lehrer, S. S., Pyrene excimer fluorescence in rabbit skeletal alpha-alpha-tropomyosin labeled with N-(1-pyrene) maleimide, *J. Biol. Chem.*, 253, 3757, 1978.
248. Graceffa, P. and Lehrer, S. S., The excimer fluorescence of pyrene-labeled tropomyosin, *J. Biol. Chem.*, 255, 11296, 1980.
249. Parry, D. A. D., Structural studies on the tropomyosin/troponin complex of vertebrate skeletal muscle, *Biochem. Biophys. Res. Commun.*, 57, 216, 1974.
250. Caspar, D. L. D., Cohen, C., and Longley, W., Tropomyosin: crystal structure, polymorphism and molecular interactions, *J. Mol. Biol.*, 41, 87, 1969.
251. Cohen, C., Caspar, D. L. D., Johnson, J. P., Nauss, K., Margossian, S. S., and Parry, D. A. D., Tropomyosin-troponin assembly, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 287, 1972
252. Cohen, C., Caspar, D. L. D., Parry, D. A. D., and Lucas, R. M., Tropomyosin crystal dynamics, *Cold Spring Harbor Symp. Quant. Biol.*, 36, 205, 1971.
253. Phillips, G. N., Jr., Lattman, E. E., Cummins, P., Lee, K. Y., and Cohen, C., Crystal structure and molecular interactions of tropomyosin, *Nature (London)*, 278, 413, 1979.
254. Cohen, C., Vibert, P. J., and Phillips, G. N., Jr., Protein switches in muscle contraction, in *Biological Recognition and Assembly*, Alan R. Liss, New York, 1979.
255. Phillips, G. N., Jr., Fillers, J. P., and Cohen, C., Motions of tropomyosin crystal as metaphor, *Biophys. J.*, 32, 485, 1980.
256. Huxley, H. E. and Brown, W. J., The low-angle X-ray diagram of vertebrate striated muscle and its behavior during contraction and rigor, *J. Mol. Biol.*, 30, 282, 1967.
257. Wahl, P., Tawada, K., and Auchet, J.-C., Study of tropomyosin labeled with a fluorescent probe by pulse fluorimetry in polarized light, *Eur. J. Biochem.*, 88, 421, 1978.
258. Mihashi, K., Thermal modification of structure of tropomyosin. II. Fluorescence depolarization of tropomyosin-fluorescein isothiocyanate conjugate; a change in the rotational relaxation time near physiological temperature, *J. Biochem. (Tokyo)*, 71, 607, 1972.
259. Chao, Y.-Y. H. and Holtzer, A., Spin-label studies of tropomyosin, *Biochemistry*, 14, 2164, 1975.
259a. Graceffa, P. and Lehrer, S., The two conformational states of tropomyosin. A spin label study, *Biophys. J.*, 37, 37a, 1983.
260. Satoh, A. and Mihashi, K., Thermal modification of structure of tropomyosin, I. Changes in the intensity and polarization of the intrinsic fluorescence (tyrosine), *J. Biochem. (Tokyo)*, 71, 597, 1972.
261. Woods, E. F., Studies on the denaturation of tropomyosin and light meromyosin, *Protein Res.*, 1, 29, 1969.
262. Pont, M. J. and Woods, E. F., Denaturation of tropomyosin by guanidine hydrochloride, *Int. J. Protein Res.*, 3, 177, 1971.
263. Woods, E. F., The conformational stabilities of tropomyosins, *Aust. J. Biol. Sci.*, 29, 405, 1976.
264. Edwards, B. F. P. and Sykes, B. D., Assignment and characterization of the histidine resonances in the H nuclear magnetic resonance spectra of rabbit tropomyosins, *Biochemistry*, 17, 684, 1978.
265. Lehrer, S. S., Effects of an interchain disulfide bond on tropomyosin structure: intrinsic fluorescence and circular dichroism studies, *J. Mol. Biol.*, 118, 209, 1978.
266. Lehrer, S. S., Graceffa, P., and Betteridge, D., Conformational dynamics of tropomyosin in solution: evidence for two conformational states, *Ann. N. Y. Acad. Sci.*, 366, 285, 1981.
267. Johnson, P. and Smillie, L. B., Polymerizability of rabbit skeletal tropomyosin: effects of enzymic and chemical modifications, *Biochemistry*, 16, 2264, 1977.
268. Mak, A. S. and Smillie, L. B., Non-polymerizable tropomyosin: preparation, some properties and F-actin binding, *Biochem. Biophys. Res. Commun.*, 101, 208, 1981.
269. Trueblood, C. E., Walsh, T. P., and Weber A., The effect of disrupting the tropomyosin strand on tropomyosin dependent functions, *Biophys. J.*, 33, 236a, 1981.
270. Pato, M. D., Mak, A. S., and Smillie, L. B., Fragments of rabbit striated muscle alpha-tropomyosin. I. Preparation and characterization, *J. Biol. Chem.*, 256, 593, 1981.
271. Pato, M. D., Mak, A. S., and Smillie, L. B., Fragments of rabbit striated muscle alpha-tropomyosin. II. Binding to troponin T, *J. Biol. Chem.*, 256, 602, 1981.
272. Fraser, R. D. B., MacRae, T. P., and Miller, A., X-ray diffraction patterns of alpha-fibrous proteins, *J. Mol. Biol.*, 14, 432, 1965.

273. Stewart, M. and McLachlan, A. D., Fourteen actin-binding sites on tropomyosin?, *Nature (London)*, 257, 331, 1975.
274. Elzinga, M., Collins, J. H., Kuehl, W. M., and Adelstein, R. S., The complete amino acid sequence of rabbit skeletal muscle actin, *Proc. Natl. Acad. Sci. U.S.A.*, 70, 2687, 1973.
275. Gergely, J. and Kohler, H., Light scattering studies on the stepwise formation and dissociation of actomyosin, in *Conf. Chemistry of Muscular Contraction*, Igaku Shoin, Tokyo, 1957, 14.
276. Hama, H., Maruyama, K., and Noda, H., Direct isolation of F-actin from myofibrils and its physicochemical properties, *Biochim. Biophys. Acta*, 102, 1945, 1965.
277. Maruyama, K., A new protein factor hindering network formation of F-actin in solution, *Biochim. Biophys. Acta*, 94, 208, 1965.
278. Maruyama, K., Some physio-chemical properties of beta-actinin. Actin factor isolated from striated muscle, *Biochim. Biophys. Acta*, 102, 542, 1965.
279. Strzelecka-Golaszewska, H., Relative affinities of divalent cations to the site of the tight calcium binding to G-actin, *Biochim. Biophys. Acta*, 310, 60, 1973.
280. Strzelecka-Golaszewska, H., Prochniewicz, E., and Drabikowski, W., Interaction of actin with divalent cations. II. Characterization of protein-metal complexes, *Eur. J. Biochem.*, 88, 229, 1978.
281. Frieden, C., Lieberman, D., and Gilbert, H. R., A fluorescent probe for conformational changes in skeletal muscle G-actin, *J. Biol. Chem.*, 255, 8991, 1980.
282. Engel, J., Fasold, H., Hulla, F. W., Waechter, F., and Wegner, A., The polymerization of muscle actin, *Mol. Cell. Biochem.*, 18, 3, 1977.
283. Wegner, A., The mechanism of ATP hydrolysis by polymer actin, *Biophys. Chem.*, 7, 51, 1977.
284. Barany, M., Finkelman, F., and Therattil-Antony, T., Studies on the bound calcium of actin, *Arch. Biochem. Biophys.*, 98, 28, 1962.
285. Martonosi, A., Gouvea, M. A., and Gergely, J., The interaction of C-labelled adenine nucleotides with actin, *J. Biol. Chem.*, 235, 1700, 1960.
286. Martonosi, A., Molino, C. M., and Gergely, J., The binding of divalent cations to actin, *J. Biol. Chem.*, 239, 1057, 1964.
287. Strzelecka-Golaszewska, H., Nagy, B., and Gergely, J., Changes in conformation and nucleotide binding of Ca, Mn, or Mg- G-actin upon removal of the bound divalent cation, *Arch. Biochem. Biophys.*, 161, 559, 1974.
288. Loscalzo, J. and Reed, G. H., Spectroscopic studies of actin-metal-nucleotide complexes, *Biochemistry*, 15, 5407, 1976.
289. Jacobson, G. R. and Rosenbusch, J. P., ATP binding to a protease-resistant core of actin, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 2742, 1976.
290. Barden, J. A., Cooke, R., Wright, P. E., and Dos Remedios, C. G., Proton nuclear magnetic resonance and electron paramagnetic resonance studies on skeletal muscle actin indicate that the metal and nucleotide binding sites are separate, *Biochemistry*, 19, 5912, 1980.
291. Martonosi, A., The specificity of the interaction of ATP with G-actin, *Biochim. Biophys. Acta*, 57, 163, 1962.
292. Drabikowski, W. and Strzelecka-Golaszewska, H., The exchange of actin-bound calcium with various bivalent cations, *Biochim. Biophys. Acta*, 71, 488, 1963.
293. Iyengar, M. R. and Weber, H. H., The relative affinities of nucleotides to G-actin and their effects, *Biochim. Biophys. Acta*, 86, 543, 1964.
294. Oosawa, F., Asakura, S., Asai, H., Kasai, M., Kobayashi, S., Mihashi, K., Ooi, F., Taniguchi, M., and Nakano, E., Structure and function of actin polymers, in *Biochemistry of Muscle Contraction*, Gergely, J., Ed., Little, Brown, Boston, 1964, 158.
295. Nagy, B. and Jencks, W. P., Optical rotatory dispersion for G-actin, *Biochemistry*, 1, 987, 1962.
296. Lehrer, S. S. and Kerwar, G., The intrinsic fluorescence of actin, *Biochemistry*, 11, 1211, 1972.
296a. Dos Remedios, C. G. and Dickens, M. J., Actin microcrystals and tubes formed in the presence of gadolinium ions, *Nature (London)*, 276, 731, 1978.
296b. Aebi, U., Fowler, W. E., Isenberg, G., Pollard, T. D., and Smith, P. R., Crystalline actin sheets: their structure and polymorphism, *J. Cell. Biol.*, 91, 340, 1981.
296c. Smith, P. R., Fowler, W. E., Pollard, T. E., and Aebi, U., Structure of the actin molecule determined from the electron micrographs of crystalline actin sheets with a tentative alignment of the molecule in the actin filament, *J. Mol. Biol.*, 167, 641, 1983.
296d. Mendelson, R., Gebhard, W., Holmes, K. C., Kabsch, W., Suck, D., Couch, J., Morris, E., and O'Brien, E., Fitting the actin monomer into the actin helix: a combination of X-ray diffraction and electron microscope data, *Biophys. J.*, 45, 391a, 1984.
296e. O'Brien, E. J., Couch, J., Johnson, G. R. P., and Morris, E. P., Structure of actin and thin filament, in *Actin, Its Structure and Function in Muscle and Non-muscle Cells*, Dos Remedios, C. and Barden, J., Eds., Academic Press, Sydney, 1983.
296f. Egelman, E. H. and DeRosier, D. J., Structural studies of F-actin, in *Actin, Its Structure and Function in Muscle and Non-muscle Cells*, Dos Remedios, C. and Barden, J., Academic Press, Sydney, 1983, 17.

296g. Tilney, L. G., Egelman, E. H., DeRosier, D. J., and Sanders, J. C., Actin filaments, stereocilia, and hair cells of the bird cochlea. II. Packing of actin filaments in the stereocilia and in the cuticular plate and what happens to the organization when the stereocilia are bent, *J. Cell. Biol.*, 96, 822, 1983.

296h. Egelman, E. H. and DeRosier, D. J., A model for F-actin derived from image analysis of isolated filaments, *J. Mol. Biol.*, 166, 623, 1983.

296j. Egelman, E. H. and Padron, R., X-ray diffraction evidence that actin is a 100 Å filament, *Nature (London)*, 307, 56, 1984.

296k. Fowler, W. E. and Aebi, U., A consistent picture of the actin filament related to the orientation of the actin molecule, *J. Cell Biol.*, 97, 264, 1983.

296m. Fujime, S., Quasi-elastic light scattering from solutions of macromolecules. II. Doppler broadening of light scattered from solutions of semi-flexible polymers, F-actin, *J. Phys. Soc. Jpn.*, 29, 751, 1970.

296n. Ishiwata, S. and Fujime, S., Effect of calcium ions on the flexibility reconstituted thin filaments of muscle studied by quasi-elastic scattering of laser light, *J. Mol. Biol.*, 68, 511, 1972.

296p. Takebayashi, T., Morita, T., and Oosawa, F., Electron microscopic investigation of the flexibility of F-actin, *Biochim. Biophys. Acta*, 492, 357, 1977.

296q. Yanagida, T. and Oosawa, F., Polarized fluorescence from epsilon-ADP incorporated into F-actin in a myosin-free single fiber: conformation of F-actin and changes induced in it by heavy meromyosin, *J. Mol. Biol.*, 126, 507, 1978.

296r. Nagashima, H. and Asakura, S., Dark-field light microscopic study of the flexibility of F-actin complexes, *J. Mol. Biol.*, 136, 169, 1980.

296s. O'Brien, E. J., Gillis, J. M., and Crouch, J., Symmetry and molecular arrangement in paracrystals of reconstituted muscle thin filaments, *J. Mol. Biol.*, 99, 461, 1975.

296t. DeRosier, D. J. and Censullo, R., Structure of F-actin needles from extracts of sea urchin oocytes, *J. Mol. Biol.*, 146, 77, 1981.

296u. Huxley, H. E. and Brown, W., The low angle X-ray diagram of vertebrate striated muscle and its behaviour during contraction and rigor, *J. Mol. Biol.*, 30, 383, 1967.

296v. Yanagida, T. and Oosawa, F., Conformational changes of F-actin-epsilon-ADP in thin filaments in myosin-free muscle fibers induced by Ca^{2+}, *J. Mol. Biol.*, 140, 313, 1980.

297. Straub, F. B. and Feuer, G., Adenosinetriphosphate: the functional group of actin, *Biochim. Biophys. Acta*, 4, 455, 1950.

298. Mommaerts, W. F. H. M., The molecular transformation of actin. III. The participation of nucleotides, *J. Biol. Chem.*, 198, 469, 1952.

299. Oosawa, F., Asakura, S., Hotta, K., Imai, N., and Ooi, T., G-F transformation of actin as a fibrous condensation, *J. Polymer Sci.*, 37, 323, 1959.

300. Asakura, S., Kasai, M., and Oosawa, F., The effect of temperature on the equilibrium state of actin solutions, *J. Polymer Sci.*, 44, 35, 1960.

301. Oosawa, F. and Kasai, M., Actin, in *Subunits in Biological Systems*, Timasheff, S. and Fasman, G., Eds., Marcel Dekker, New York, 1971, pgs. 5 and 261.

302. Oosawa, F. and Asakur, S., *Thermodynamics of the Polymerization of Protein*, Academic Press, London, 1975.

303. Wegner, A., Head on microscopy of actin filaments nucleated by isolated microvillus cores, *J. Cell. Biol.*, 80, 654, 1981.

304. Hill, T. L., Bioenergetic aspects and polymer length distribution in steady-state head-to-tail polymerization of actin or microtubules, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 4803, 1980.

304a. Hill, T. L. and Kirschner, M. W., Bioenergetics and kinetics of microtubule and actin filament assembly-disassembly, *Int. Rev. Cytol.*, 78, 1, 1982.

305. Pollard, T. D. and Mooseker, M. S., Direct measurement of actin polymerization rate constants by electron microscopy of actin filaments nucleated by isolated microvillus cores, *J. Cell. Biol.*, 80, 654, 1981.

306. Wegner, A. and Neuhaus, J.-M., Requirement of divalent cations for fast exchange of actin monomers and actin filament subunits, *J. Mol. Biol.*, 153, 681, 1981.

307. Wegner, A., Treadmilling of actin at physiological salt concentration, *J. Mol. Biol.*, 161, 607, 1982.

308. Wegner, A. and Engel, J. Kinetics of the cooperative association of actin to actin filament, *Biophys. Chem.*, 3, 215, 1975.

309. Hayashi, T. and Ip, W., Polymerization polarity of actin, *J. Mechanochem. Cell Motil.*, 3, 163, 1976.

310. Woodrum, D. T., Rich, S. A., and Pollard, T. D., Evidence for biased bidirectional polymerization of actin filaments using heavy meromyosin prepared by an improved method, *J. Cell Biol.*, 67, 231, 1975.

311. Kondo, H. and Ishiwata, S., Uni-directional growth of F-actin, *J. Biochem. (Tokyo)*, 79, 159, 1976.

312. Mockrin, S. C. and Korn, E. D., Isolation and characterization of covalently crosslinked actin dimer, *J. Biol. Chem.*, 256, 8288, 1981.

313. Brenner, S. L. and Korn, E. D., The effects of cytochalasins on actin polymerization and actin ATPase provide insights into the mechanism of polymerization, *J. Biol. Chem.*, 255, 841, 1980.
314. Pardee, J. D. and Spudich, J. A., Mechanism of K-induced actin assembly, *J. Cell. Biol.*, 87, 226a, 1980.
315. Rich, S. A. and Estes, J. E., Detection of conformational changes in actin by proteolytic digestion: evidence for a new monomeric species, *J. Mol. Biol.*, 104, 777, 1976.
316. Rouayrenc, J.-F. and Travers, F., The first step in the polymerization of actin, *Eur. J. Biochem.*, 116, 73, 1981.
317. Kouyama, T. and Mihashi, K., Pulse-fluorometry study on actin and heavy meromyosin using F-actin labelled with N-(1-pyrene) maleimide, *Eur. J. Biochem.*, 105, 279, 1980.
318. Asakura, S., Taniguchi, M., and Oosawa, F., The effect of sonic vibration on the exchangeability and reactivity of the bound adenosine triphosphatase of F-actin, *Biochim. Biophys. Acta*, 74, 140, 1963.
319. Wegner, A. and Savko, P., Fragmentation of actin filaments, *Biochemistry*, 21, 1909, 1982.
320. Wegner, A., Spontanteous fragmentation of actin filaments in physiological conditions, *Nature (London)*, 96, 266, 1982.
321. Cooke, R. and Murdoch, L., Interaction of actin with analogs of adenosine triphosphate, *Biochemistry*, 12, 3927, 1973.
322. Cooke, R., The role of the bound nucleotide in the polymerization of actin, *Biochemistry*, 14, 3250, 1975.
322a. Brenner, S. L. and Korn, E. D., On the mechanism of actin monomer-polymer exchange at steady state, *J. Biol. Chem.*, 258, 5013, 1983.
323. Kirschner, M. W., Implications of treadmilling for the stability and polarity of actin and tubulin polymers in vivo, *J. Cell Biol.*, 86, 330, 1980.
324. Taylor, D. L., Reidler, J., Spudich, J. A., and Stryer, L., Detection of actin assembly by fluorescence energy transfer, *J. Cell. Biol.*, 89, 362, 1981.
325. Kikuchi, M., Noda, H., and Maruyama, K., Interaction of actin within H-meromyosin at low ionic strength, *J. Biochem. (Tokyo)*, 65, 945, 1969.
326. Wegner, A., Kinetic analysis of actin assembly suggests that tropomyosin inhibits spontaneous fragmentation of actin filaments, *J. Mol. Biol.*, 161, 217, 1982.
327. Hama, H., Maruyama, K., and Noda, H., Direct isolation of F actin from myofibrils and its physiochemical properties, *Biochim. Biophys. Acta*, 102, 149, 1965.
328. Maruyama, K., A study of beta-actinin, myofibrillar protein from rabbit skeletal muscle, *J. Biochem. (Tokyo)*, 69, 369, 1971.
329. Kawamura, M. and Maruyama, K., Length distribution of F-actin transformed from Mg-polymer, *Biochim. Biophys. Acta*, 267, 422, 1972.
330. Carlsson, L., Nystrom, L.-E., Sundkvist, I., Markey, F., and Lindberg, J., Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells, *J. Mol. Biol.*, 115, 465, 1977.
331. Isenberg, G., Aebi, U., and Pollard, T. D., An actin-binding protein from Acanthamoeba regulates actin filament polymerization and interactions, *Nature (London)*, 288, 455, 1980.
332. Hitchcock-DeGregori, S. E., Actin assembly, *Nature (London)*, 288, 437, 1980.
333. Schliiwa, M., Proteins associated with cytoplasmic actin, *Cell*, 25, 57, 1981.
334. Korn, E. D., Biochemistry of actomyosin-dependent cell motility, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 588, 1978.
335. Kuschinsky, G. and Turba, F., Uber die rolle de SH-gruppen bei vorgangen am aktomyosin, myosin and aktin, *Biochim. Biophys. Acta*, 6, 426, 1951.
336. Drabikowski, W. and Gergely, J., The role of sulfhydryl groups in the polymerization and adenosine triphosphate binding of G actin, *J. Biol. Chem.*, 238, 640, 1963.
337. Bailin, G. and Barany, J. Studies on actin-actin and actin-myosin interactions, *Biochim. Biophys. Acta*, 14, 208, 1967.
338. Elzinga, M. and Collins, J. H., The amino acid sequence of rabbit skeletal muscle actin, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 1, 1972.
339. Lusty, C. J. and Fasold, H., Characterization of sulfhydryl groups of actin, *Biochemistry*, 8, 2933, 1969.
340. Lehrer, S. S., Nagy, B., and Gergely, J., The binding of copper to actin without loss of polymerizability. The involvement of the rapidly reacting SH groups, *Arch. Biochem. Biophys.*, 150, 164, 1972.
341. Tao, T. and Cho, J., Fluorescence lifetime quenching studies on the accessibilities of actin sulfhydryl sites, *Biochemistry*, 18, 2759, 1979.
342. Cheung, H., Cooke, R., and Smith, L., The G-actin-F-actin transformation as studied by the fluorescence of bound dansyl cystine, *Arch. Biochem. Biophys.*, 142, 333, 1971.

343. Ando, T. and Asai, H., Conformational change in actin filament induced by the interaction with heavy meromyosin: effects of pH, tropomyosin and deoxy-ATP, *J. Mol. Biol.*, 129, 265, 1979.
343a. Leavis, P. C. and Lehrer, S. S., A sulfhydryl-specific fluorescent label, S-mercuric-N-dansylcysteine. Titrations of glutathione and muscle proteins, *Biochemistry*, 13, 3042, 1974.
344. Stone, D. B., Prevost, S. C., and Botts, J., Studies on spin labeled actin, *Biochemistry*, 9, 3937, 1970.
345. Burley, R., Seidel, J. C., and Gergely, J., The stoichiometry of the reaction of the spin labeling of F actin and the effect of orientation of spin labeled F actin filaments, *Arch. Biochem. Biophys.*, 146, 597, 1971.
346. Burley, R., Seidel, J. C., and Gergely, J., The effect of divalent metal binding on the electron spin resonance spectra of spin labeled actin. Evidence of a spin-spin interaction involving Mn, *Arch. Biochem. Biophys.*, 150, 792, 1972.
347. Thomas, D. D., Seidel, J. C., and Gergely, J., Rotational dynamics of spin labelled F actin and in the submillisecond time range, *J. Mol. Biol.*, 132, 257, 1979.
348. Duke, J., Takashi, R., Eu, K., and Morales, M. F., Reciprocal reactivities of specific thiols when actin binds to myosin, *Proc. Natl. Acad. Sci. U.SA.*, 73, 302, 1976.
349. Miki, M., Conformational change in reconstituted thin filament studied with fluorescence energy transfer between ε-ATP bound to F-actin and NBD-Cl bound to troponin C, *Biochim. Biophys. Acta*, 578, 96, 1979.
350. Lu, R. C. and Szilagyi, L., Change of reactivity of lysine residues upon actin polymerization, *Biochemistry*, 20, 5914, 1981.
351. Ohnishi, S., Maruyama, K., and Ebashi, S., Calcium-induced conformational changes and mutual interactions of troponin components as studied by spin-labeling, *J. Biochem. (Tokyo)*, 78, 73, 1975.
352. Leavis, P. C. and Tao, T., A fluorescent quenching study of troponin subunit interaction, *Fed. Proc.*, 39, 724, 1980.
353. Sutoh, K. and Matsuzaki, F., Millisecond photo-cross-linking of protein components in vertebrate striated muscle thin filaments, *Biochemistry*, 19, 3878, 1980.
354. Cox, J. A., Comte, M., and Stein, E. A., Calmodulin-free skeletal-muscle troponin C prepared in the absence of urea, *Biochem. J.*, 195, 205, 1981.
355. Zot, H. G. and Potter, J. D., A structural role for the Ca^{2+}-Mg^{2+} sites on troponin C in the regulation of muscle contraction, *J. Biol. Chem.*, 257, 7678, 1982.
356. Grabarek, Z., Leavis, P. C., and Gergely, J., Kinetics of dissociation of complexes of troponin C, *Fed. Proc.*, 40, 1742, 1981.
357. Leavis, P. C., A fluorescent change in the troponin C-troponin I complex upon calcium binding to the calcium-specific binding sites, *Fed. Proc.*, 35, 1746, 1976.
358. Strong, L. H., Fossel, E. T., and Gergely, J., F-NMR studies of calcium induced changes in troponin, *Biophys. J.*, 17, 118a, 1977.
359. Johnson, J. D., Robertson, S. P., and Potter, J. D., Ca^{2+}-exchange rates of the Ca^{2+}-Mg^{2+} and the Ca^{2+}specfic sites of whole troponin, *Fed. Proc.*, 39, 1621, 1980.
360. Trybus, K. M. and Taylor, E. W., Kinetic studies of the cooperative binding of subfragment-1 to regulated actin, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 7209, 1981.
361. van Eerd, J. P. and Kawasaki, Y., Effect of calcium (II) on the interaction between the subunits of troponin and tropomysin, *Biochemistry*, 12, 4972, 1973.
362. Gusev, N. B. and Friedrich, P., Calcium-induced conformational changes in the troponin complex detected by cross-linking, *Biochim. Biophys. Acta*, 626, 106, 1980.
363. Pearlstone, J. R. and Smillie, L. B., Binding of troponin T fragments to several types of tropomyosin, *J. Biol. Chem.*, 257, 10587, 1982.
364. Chong, P. C. S. and Hodges, R. S., Photochemical crosslinking between rabbit skeletal troponin and alpha-tropomyosin, *J. Biol. Chem.*, 257, 9152, 1982.
365. Katayama, E., Interaction of troponin I with troponin T and its fragments, *J. Biochem (Tokyo)*, 85, 1379, 1979.
366. Moore, P. B., Huxley, H. E., and DeRosier, D. J., Three dimensional reconstruction of F-actin, thin filaments and decorated thin filaments, *J. Mol. Biol.* 50, 279, 1970.
367. O'Brien, E. J., Bennet, P. M., and Hanson, J., Optical diffraction studies of myofibrillar structure, *Philos. Trans. R. Soc. London Ser. B*, 261, 201, 1971.
368. Spudich, J. A., Huxley, H. E., and Finch, J. T., Regulation of skeletal muscle contraction. II. Structural studies of the interaction of the tropomyosin-troponin complex with actin, *J. Mol. Biol.*, 72, 619, 1973.
369. Huxley, H. E., Structural changes in the actin- and myosin-containing filaments of vertebrate striated muscle, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 361, 1972.
370. Haselgrove, J. C., X-ray evidence for a conformational change in the actin-containing filaments of vertebrate striated muscle, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 341, 1972.

371. Parry, D. A. D. and Squire, J. M., Structural role of tropomyosin in muscle regulation: analysis of the X-ray diffraction patterns from relaxed and contracting muscles, *J. Mol. Biol.*, 75, 33, 1973.
372. Wakabayashi, T., Huxley, H. E., Amos, L. A., and Klug, A., Three-dimensional image reconstruction of actin-tropomyosin complex and actin-tropomyosin-troponin T-troponin I complex, *J. Mol. Biol.*, 93, 477, 1975.
373. Seymour, J. and O'Brien, E. J., The position of tropomyosin in muscle thin filaments, *Nature (London)*, 283, 680, 1980.
374. Taylor, K. A. and Amos, L. A., A new model for the geometry of the binding of myosin crossbridges to muscle thin filaments, *J. Mol. Biol.*, 147, 297, 1981.
375. Wegner, A., Equilibrium of the actin-tropomyosin interaction, *J. Mol. Biol.*, 131, 839, 1979.
376. McGhee, J. D. and von Hippel, P. H., Theoretical aspects of DNA-protein interactions: cooperative and non-cooperative binding of large ligands to a one-dimensional homogeneous lattice, *J. Mol. Biol.*, 86, 469, 1974.
377. Trueblood, C. E., Walsh, T. P., and Weber, A., The effect of disrupting the tropomyosin strand on tropomyosin dependant functions, *Biophys. J.*, 33, 236a, 1981.
377a. Uen, H., Tawada, Y., and Ooi, T., Properties of non-polymerizable tropomyosin obtained by carboxypeptidase A digestion, *J. Biochem. (Tokyo)*, 80, 283, 1976.
378. Drabikowski, W., Kominz, D. R., and Maruyama, K., Effect of troponin on the reversibility of tropomyosin binding to F-actin, *J. Biochem. (Tokyo)*, 63, 802, 1968.
379. Fujime, S. and Ishiwata, S., Dynamic study of F-actin by quasielastic scattering of laser light, *J. Mol. Biol.*, 62, 251, 1972.
380. Yang, Y., Korn, E. D., and Eisenberg, E., Cooperative binding of tropomyosin to muscle and Acanthamoeba actin, *J. Biol. Chem.*, 254, 7137, 1979.
381. Drabikowski, W. and Nowak, E., Studies on the interaction of F-actin with tropomyosin, *Eur. J. Biochem.*, 5, 376, 1968.
382. Spudich, J. A. and Watt, S., The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin, *J. Biol. Chem.*, 246, 4866, 1971.
383. Tanaka, H. and Oosawa, F., The effect of temperature on the interaction between F-actin and tropomyosin, *Biochim. Biophys. Acta*, 253, 274, 1971.
384. Walsh, T. P. and Wegner, A., Effect of the state of oxidation of cysteine 190 of tropomyosin on the assembly of the actin-tropomyosin complex, *Biochim. Biophys. Acta*, 626, 79, 1980.
385. Wegner, A., The interaction of alpha, alpha- and alpha, beta-tropomyosin with actin filaments, *FEBS Lett.*, 91, 237, 1978.
386. Cote, C. P. and Smillie, L. B., The interaction of equine platelet tropomyosin with skeletal muscle actin, *J. Biol. Chem.*, 256, 7257, 1981.
387. Cote, P., Lewis, W. G., and Smillie, L. B., Nonpolymerizability of platelet tropomyosin and its NH and COOH-terminal sequences, *FEBS Lett.*, 91, 237, 1978.
388. Eaton, B. L., Tropomyosin binding to F-actin induced by myosin heads, *Science*, 192, 1337, 1976.
389. Wegner, A. and Walsh, T. P., Interaction of tropomyosin-troponin with actin filaments, *Biochemistry*, 20, 5633, 1981.
390. Hitchcock, S. E., Regulation of muscle contraction: binding of troponin and its components to actin and tropomyosin, *Eur. J. Biochem.*, 52, 255, 1975.
391. Katayama, E., Interactions among the thin filament proteins, in *Muscle Contraction: Its Regulatory Mechanisms*, Ebashi, S., Maruyama K., and Endo, M., Eds., Japan Scientific Societies Press, Tokyo, 1980, 251.
392. Hitchcock, S. E., Regulation of muscle contraction. Effect of calcium on the affinity of troponin for actin and tropomyosin, *Biochemistry*, 12, 2509, 1973.
393. Hitchock, S. E., Regulation of muscle contraction: binding of troponin and its components to actin and tropomyosin, *Eur. J. Biochem.*, 52, 255, 1975.
394. Hitchcock, S. E., Huxley, H. E., and Szent-Gyorgyi, A. G., Calcium sensitive binding of troponin to actin-tropomyosin: a two-site model for troponin action, *J. Mol. Biol.*, 80, 825, 1973.
395. Eaton, B. L., Kominz, D. R., and Eisenberg, E., Correlation between the inhibition of the actoheavy meromyosin ATPase and the binding of tropomyosin to F-actin: effects of Mg^{2+}, KCl, troponin-I and troponin-C, *Biochemistry*, 14, 2718, 1975.
396. Drabikowski, W. and Nonomura, Y., The interaction of troponin with F-actin and its abolition by tropomyosin, *Biochim. Biophys. Acta*, 60, 129, 1968.
397. Fuji, T., Kawamura, M., Yamamoto, K., and Maruyama, K., Interaction of troponin I component and F-actin, *J. Biochem. (Tokyo)*, 73, 269, 1973.
398. Johnson, P. and Stockmal, V. B., Evidence for actin-troponin-T interaction at physiological ionic strength, *Int. J. Biol. Macromol.*, 2, 384, 1980.
399. Grabarek, Z., Grabarek, J., Gergely, J., and Leavis, P. C., Cooperative interactions in the Ca^{2+}-dependent activation of actomyosin ATPase, *Biophys. J.*, 33, 147a, 1981.

400. Tonomura, Y., Watanabe, S., and Morales, M., Conformational changes in the molecular control of muscle contraction, *Biochemistry*, 8, 2171, 1969.
401. Yanagida, T., Taniguchi, M., and Oosawa, F., Conformational changes of F-actin in the thin filaments of muscle induced in vivo and in vitro by calcium ions, *J. Mol. Biol.*, 90, 509, 1974.
402. Yanagida, T. and Oosawa, F., Conformational changes of F-actin-epsilon-ADP in thin filaments in myosin-free muscle fibers induced by Ca^{2+}, *J. Mol. Biol.*, 140, 313, 1980.
403. Knight, P. and Offer, G., Investigation, by cross-linking, of conformational changes in F-actin during its interaction with myosin, *Biochemistry*, 19, 4682, 1980.
404. Thomas, D. D., Seidel, J. C., and Gergely, J., Rotational dynamics of spin-labeled F-actin in the sub-millisecond time range, *J. Mol. Biol.*, 132, 257, 1979.
405. Poo, W.-J. and Hartshorne, D. J., Actin cross-linked with glutaraldehyde: evidence to suggest an active role for actin in the regulatory mechanism, *Biochem. Biophys. Res. Commun.*, 70, 406, 1976.
406. Prochniewicz, E., Effect of crosslinking by glutaraldeyde on interaction of F-actin with heavy meromyosin, *Biochim. Biophys. Acta*, 579, 346, 1979.
406a. Lehrer, S. S., The crosslinking of actin and tropomyosin by glutaraldehyde, *Biochem. Biophys. Res. Commun.*, 48, 967, 1972.
407. Mikawa, T., "Freezing" of the calcium-regulated conformation of reconstituted thin filament of skeletal muscle by glutaraldehyde, *Nature (London)*, 278, 473, 1979.
408. Tao, T. and Morris, E., Computer simulation study on the extent of energy transfer from a single Tm-bound donor to multiple actin-bound acceptors, *Biochemistry*, 22, 3059, 1983.
409. Miki, M., Conformational change in reconstituted thin filament studied with fluorescence energy transfer between epsilon-ADP bound to F-actin and NBD-Cl bound to troponin-C, *Biochim. Biophys. Acta*, 578, 96, 1979.
410. Hanson, J., Lednev, V., O'Brien, E. J., and Bennet, P. M., Structure of actin-containing filaments in vertebrate skeletal muscle, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 311, 1972.
411. Lowy, J. and Vibert, P. J., Studies of the low-angle X-ray pattern of a molluscan smooth muscle during tonic contraction and rigor, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 353, 1972.
412. O'Brien, E. J., Morris, E. P., Seymour, J., and Couch, J., Structure and activity of the thin filament, in *Muscle Contraction: Its Regulatory Mechanisms*, Ebashi, S., Maruyama, K., and Endo, M., Eds., Japan Scientific Societies Press, Tokyo, 1980, 147.
413. Seymour, J. and O'Brien, E. J., The position of tropomyosin in muscle thin filaments, *Nature (London)*, 283, 680, 1980.
414. Huxley, H. E., Structural differences between resting and rigor muscle; evidence from intensity changes in the low-angle equatorial X-ray diagram, *J. Mol. Biol.*, 37, 507, 1968.
415. Egelman, E. H., Francis, N., and DeRosier, D. J., F-actin is a helix with a random variable twist, *Nature (London)*, 298, 131, 1982.
416. Taylor, K. A. and Amos, L. A., A new model for the geometry of the binding of myosin crossbridges to muscle thin filaments, *J. Mol. Biol.*, 147, 297, 1981.
417. Wakabayashi, T., Three-dimensional image analysis of the complex of thin filaments and myosin molecules which form skeletal muscle, in *Muscle Contraction: Its Regulatory Mechanisms*, Ebashi, S., Maruyama, K., and Endo, M., Eds., Japan Scientific Societies Press, Tokyo, 1980, 79.
418. Suck, D., Kabsch, W., and Mannherz, H. G., Three-dimensional structure of the complex of skeletal muscle actin and bovine pancreatic DNase 1 at 6-A resolution, *Proc. Natl. Acad. Sci. U.S.A.*, 78, 4319, 1981.
419. Aebi, U., Smith, P. R., Isenberg, G., and Pollard, T. D., Structure of crystalline actin sheets, *Nature (London)*, 288, 296, 1980.
420. Amos, L. A., Huxley, H. E., Holmes, K. C., Goody, R S., and Taylor, K. A., Structural evidence that myosin heads may interact with two sites on F-actin, *Nature (London)*, 299, 467, 1982.
421. Mornet, D., Bertrand, R., Pantel, P., Audemard, E., and Kassab, R., Structure of the actin-myosin interface, *Nature (London)*, 292, 301, 1981.
422. Craig, R., Szent-Gyorgyi, A. G., Beese, L., Flicker, P., Vibert, P., and Cohen, C., Electron microscopy of thin filaments decorated with a Ca^{2+}-regulated myosin, *J. Mol. Biol.*, 140, 35, 1980.
423. Vibert, P. and Craig, R., Three-dimensional reconstruction of thin filaments decorated with a Ca^{2+}-regulated myosin, *J. Mol. Biol.*, 157, 299, 1982.
424. Bremel, R. D., Murray, J. M., and Weber, A., Manifestations of cooperative behavior in the regulated actin filament during actin-activated ATP hydrolysis in the presence of calcium, *Cold Spring Harbor Symp. Quant. Biol.*, 37, 267, 1972.
425. Bremel, R. D. and Weber, A., Cooperation within actin filaments in vertebrate skeletal muscle, *Nature (London)*, 238, 97, 1972.
426. Lymn, R. W. and Taylor, E. W. Transient state phosphate production in the hydrolysis of nucleoside triphosphates by myosin, *Biochemistry*, 9, 2975, 1970.
427. Katz, A. M., Influence of tropomyosin upon the reactions of actomyosin at low ionic strength, *J. Biol. Chem.*, 239, 3304, 1964.

428. Murray, J. M., Knox, M. K., Trueblood, C. E., and Weber, A., Potentiated state of the tropomyosin-actin filament and nucleotide-containing myosin subfragment 1, *Biochemistry*, 21, 906, 1982.
429. Murray, J., M., Weber, A., and Bremel, R. D., Could cooperativity in the actin filaments play a role in muscle contraction?, in *Calcium Transport in Contraction and Secretion*, Carafoli, E., Clementi, F., Drabikowski, W., and Margreth, A., Eds., North-Holland, New York, 1975, 489.
430. Murray, J. M., Knox, M. K., Trueblood, C. E., and Weber, A., Do tropomyosin and myosin compete for actin sites in the presence of calcium?, *FEBS Lett.*, 114, 169, 1980.
431. Lehrer, S. S. and Morris, E. P., Dual effects of tropomyosin and troponin-tropomyosin on actomyosin subfragment 1 ATPase, *J. Biol. Chem.*, 251, 8073, 1982.
432. Sobieszek, A., Steady-state kinetic studies on the actin activation of skeletal muscle heavy meromyosin subfragments, *J. Mol. Biol.*, 157, 275, 1982.
433. Greene, L. E. and Eisenberg, E., Cooperative binding of myosin subfragment-1 to the actin-troponin-tropomyosin complex, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 2616, 1980.
434. Hill, T. L., Eisenberg, E., and Greene, L., Theoretical model for the cooperative equilibrium binding of myosin subfragment 1 to the actin-troponin-tropomyosin complex, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 3186, 1980.
435. Hill, T. L., Binding under a molecular umbrella as a cooperative statistical mechanical system: tropomyosin-actin-myosin as an example, *Biophys. Chem.*, 14, 31,1982.
436. Nagashima, H. and Asakura, S., Studies on co-operative properties of tropomyosin-actin and tropomyosin-troponin-actin complexes by the use of N-ethylmaleimide-treated and untreated species of myosin subfragment 1, *J. Mol. Biol.*, 155, 407, 1982.
437. Murray, J. M., Weber, A., and Knox, M. K., Myosin subfragment 1 binding to relaxed actin filaments and steric model of relaxation, *Biochemistry*, 20, 641, 1981.
438. Stein, L. A., Schwartz, R. P., Jr., Chock, P. B., and Eisenberg, E., Mechanism of actomyosin ATPase. Evidence that adenosine 5′-triphosphate hydrolysis can occur without dissociation of the actomyosin complex, *Biochemstry*, 18, 3895, 1979.
439. Stein, L. A., Chock, P. B., and Eisenberg, E., Mechanism of the actomyosin ATPase: effect of actin on the ATP hydrolysis step, *Proc. Natl. Acad. Sci. U.S.A.*, 78, 1346, 1981.
440. Chalovich, J. M., Chock, P. B., and Eisenberg, E., Mechanism of action of troponin-tropomysin. Inhibition of actomyosin ATPase activity without inhibition of myosin binding to actin, *J. Biol. Chem.*, 256, 575, 1981.
441. Chalovich, J. M. and Eisenberg, E., Inhibition of actomyosin ATPase activity without blocking the binding of myosin to actin, *J. Biol. Chem.*, 257, 2432, 1982.
442. Nagy, B., Potter, J. D., and Gergely, J., Calcium induced conformational changes in a cyanogen bromide fragment of troponin C that contains one of the binding sites, *J. Biol. Chem.*, 253, 5471, 1978.
443. Squire, J., *The Structural Basis of Muscle Contraction*, Plenum Press, New York, 1981, chap. 5.
444. McLachlan, A. D. and Stewert, M., The 14-fold periodicity in alpha-tropomyosin and the interaction with actin, *J. Mol. Biol.*, 103, 271, 1976.