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REVIEW

Tropomyosin: Double Helix from the Protein World

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Abstract—This review concerns the structure and functions of tropomyosin (TM), an actin-binding protein that plays a key role in the regulation of muscle contraction. The TM molecule is a dimer of α-helices, which form a coiled-coil. Recent views on the TM structure are analyzed, and special attention is concentrated on those structural traits of the TM molecule that distinguish it from the other coiled-coil proteins. Modern data are presented on TM functional properties, such as its interaction with actin and ability to move on the surface of actin filaments, which underlies the regulation of the actin–myosin interaction upon contraction of skeletal and cardiac muscles. Also, part of the review is devoted to analysis of the effects of mutations in TM genes associated with muscle diseases (myopathies) on the structure and functions of TM.

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Tropomyosin (TM) is one of the key components of the regulatory apparatus of thin filaments in all types of muscles. Extended TM molecules bind seven actin monomers and one troponin complex within the thin filament. Present-day concepts of the regulatory mechanism of skeletal and cardiac muscle contraction are formulated as the "theory of steric blocking" according to which TM is able to move azimuthally on the surface of the actin filament, thus opening or closing binding sites with the heads of myosin molecules.

There are four TM genes that give rise to over 30 isoforms through alternative splicing. In human skeletal muscles TM exists as a mixture of homodimers of α- and β-isoforms, while the smooth muscle contains basically αβ-heterodimers. The TM molecule is a dimer of α-helices forming a left-handed superhelix often referred to as a "coiled-coil". This is accounted for by the presence of continuous seven-membered repeats (heptads) in the TM primary structure. Due to the simplicity of its design, the TM molecule is an ideal model system for investigation of the structure–function relationship in protein molecules. In this respect, amino acid substitutions (mutations) in TM, associated with various diseases such as myopathies, are of special interest. Such mutations are usually characterized by pronounced functional phenotype; therefore, studying structural alterations in TM caused by these mutations is interesting for both fundamental science and biomedicine.

Until recently TM had been regarded as one of the most typical members of the α-helical coiled-coil protein family; moreover, it was often considered as a classic example of this class. However, a substantial body of data accumulated over the recent decades indicates that the structure of TM is not as straightforward as it had been believed. Currently, substantial attention is drawn to unconventional structural features unique to TM (e.g. conformational flexibility of some part of the molecule) that underlie its regulatory function. One of the main goals of this review is to analyze the evolution of concepts of TM structure and functions from its discovery in 1946 till now with emphasis on TM structural peculiarities that distinguish it from the other coiled-coil proteins. However, it should be mentioned that due to space limitations we shall not consider several important problems associated with TM structure and functions. Thus, we shall not be able to pay due attention to the synthesis of various TM isoforms (including alternative splicing of TM gene products). Another important question concerns the role of TM in regulation of actin cytoskeleton structure and function. And finally, numerous data are appearing in the modern literature on how alterations in synthesis of various TM isoforms are associated with malignant cell transformation. Analysis of this interesting problem is also beyond the scope of our review.

Abbreviations: CD, circular dichroism; DSC, differential scanning calorimetry; S1, myosin subfragment 1; TM, tropomyosin.

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Thus, the review represents an analysis of the results derived from many recent studies on TM structure and function, including those carried out in our laboratory by means of differential scanning calorimetry (DSC). We shall pay substantial attention to analysis of alterations of TM structure and functions caused by disease (e.g. myopathy)-associated point mutations.

TROPOMYOSIN STRUCTURE

Discovery of Tropomyosin and Initial Data on Its Structure

Tropomyosin (TM) was discovered by Bailey in 1946 [1] during treatment of myofibrils with organic solvents causing selective denaturing of myosin. Simultaneously, the asymmetrical nature of this protein was detected, despite which TM was easily crystallized with formation of fragile plates containing ~90% water. At the same time such an important property of TM solution as high viscosity at low ionic strength was noted. Electron microscopy made it possible to show that under these conditions TM forms long fibrils of approximately the same diameter, which undergo depolymerization when ionic strength of the solution increases [2]. On the basis of these and some other data, Bailey put forward a hypothesis concerning dimeric structure of the isolated protein molecule, which was later confirmed. Despite this, nothing was known about TM functions, except suggestions about its structural role based on the fibrillar nature of the protein. Based on similarity of amino acid composition and physical properties, Bailey concluded that the isolated protein was most likely the precursor of myosin and therefore it was named "tropomyosin". Although this hypothesis was proved wrong in later studies, certain progress in understanding TM structure and function was definitely achieved.

X-ray diffraction data on thin films of TM ascribed TM to a protein family that also includes α-helical α-keratin, myosin, epidermin, and fibrinogen (the so-called *k-m-e-f* class of proteins). To explain the special diffraction pattern characteristic of these proteins, in 1953 Francis Crick proposed a simple and elegant model of packing of two (or three) α-helices with the formation of superhelix or coiled-coil structure as it was named by Crick. According to this model, helices are fixed opposite one another due to hydrophobic interactions between the residues localized in the region of contact of the helices. These interactions were designated by the author as "knob into holes" because each residue in the helix contact region is surrounded by three amino acid residues belonging to the other helix. The structure of a bundle of α-helices proposed by Crick suggested the existence of periodicity in the primary structure of helices, which was one of the most important consequences of this hypothesis [3].

In 1975 Smillie et al. completed the determination of primary structure of TM from rabbit skeletal muscle. Analysis of the amino acid sequence (284 a.a.) revealed periodic arrangement of hydrophobic amino acids within the polypeptide chain. It was shown that the primary structure can be divided into seven-membered repeats, the first and fourth residues of which contain nonpolar moieties. Thus, the primary structure of TM revealed full coincidence with the coiled-coil structure predicted by Crick [4, 5]. At this step, the fact that the TM molecule is represented by two α-helices, forming a coiled-coil, was already beyond doubt. Nevertheless, it still remained unclear whether TM chains are located in register, because the model of Crick did not exclude the probability of chain shift relative to each other for the number of residues multiple by seven. In 1975 S. Lehrer showed that TM chains can be chemically "cross-linked" by disulfide bond between Cys190 residues [6]. This result showed unambiguously that TM chains are located strictly one opposite another, i.e. "in register". The structural periodicity of TM was the subject of investigations in many subsequent works. Parry as well as McLachlan and Stewart revealed periodicity in the arrangement of charged residues in the amino acid sequence of TM [7, 8]: each fifth and seventh residue in the seven-membered repeat contained a charged moiety. This observation led to the idea of existence of ionic interactions additionally stabilizing the double helix. This hypothesis was confirmed almost 20 years later when O'Shea et al. determined the atomic structure of transcription factor GCN4, which also has the coiled-coil structure [9]. To sum up the history of TM studies, we note that until the 1990s TM was considered as a prototype protein with the coiled-coil structure.

Coiled-Coil Structure: Main Features and Nomenclature

As shown by numerous proteomic investigations, the coiled-coil is a widespread structural motif. For example, the coiled-coil type structure is the basis for intermediate filaments [10]. Also, such various protein molecules like molecular motors (myosins, kinesins, and dyneins), transcription factors (such as yeast GCN4), and cell surface receptors (such as the macrophage scavenger receptor) all contain domains with the coiled-coil structure. In terms of function, these regions are usually modules that mediate oligomerization. A good example of this is formation of heterodimers of transcription factors *c-jun* and *c-fos* by means of coiled-coil structure formation [11]. It is noteworthy that disruption of this process results in a number of consequences including cancer transformation of the cell.

Let us consider in more detail the basic features of the coiled-coil structure. It usually consists of two-to-five right-handed α-helices that form a unique left-handed

superhelix termed coiled-coil. In this case the axes of the α-helices are not parallel but shifted relative each other by 20°. As noted earlier, primary structure of the coiled-coil type proteins is characterized by periodicity: the amino acid sequence of these proteins is organized in repeating motives (heptads) of seven amino acid residues (Fig. 1A; see color insert) (the similarity in amino acid composition of tropomyosin and core part of myosin revealed by Bailey follows from this). Unlike single α-helices in which 3.6 amino acid residues advance per one turn, coiled-coil is characterized by 3.5 residues per turn due to conformational limitations in α-helices caused by dimer formation. This means that in the coiled-coil structure two helix turns correspond to one heptade repeat. In heptads each amino acid is designated by a Latin letter (*abcdefg*). Arndt et al. put forward the so-called PV-hypothesis ("Peptide Velcro") summing up structural requirements necessary for formation of specific coiled-coil structure [12]. First of all, the hypothesis requires the presence in positions *a* and *d* of hydrophobic residues (valine, leucine, isoleucine) able to stabilize the coiled-coil structure by hydrophobic and van der Waals interactions. Also, positions *e* and *g* should be occupied by amino acids with charged side chains, able to form stabilizing salt bridges between adjacent helices. The remaining positions *b*, *c*, and *f* are ascribed to residues with polar radicals because in the structure of α-helix they are in direct contact with the solvent.

As indicated above, interactions between residues *a* and *d* were predicted by Crick and called "knob into holes". The point is that each residue at the interface of two helices is localized in the cavity formed by three residues belonging to the opposite helix (Fig. 1, B and C). Such interactions result in formation of hydrophobic "core" of coiled-coil, which is similar to formation of hydrophobic nucleus in the case of globular protein folding. Similarly to the globular proteins, hydrophobic interactions appear to be the main determinants of molecular stability. Hodges et al. noted that the most stable coiled-coil is characterized by the highest occurrence of hydrophobic residues in positions *a* and *d* [13]. Investigation of the stabilizing properties of various *a*- and *d*-localized residues in the model dimeric coiled-coils revealed that the highest stabilizing effect is exhibited by hydrophobic residues with intermediate size side chains (Leu, Ile, Val). Then there follow hydrophobic residues with bulky side chains (Tyr, Trp), alanine residues, uncharged hydrophilic residues (Gln, Asn, Ser), positively charged residues (Arg, Lys), negatively charged residues (Glu, Asp), glycine, and proline [14, 15]. Moreover, it was shown that incorporation of unnatural amino acids with increased hydrophobicity (such as 5,5,5-trifluoroleucine) into hydrophobic core of model coiled-coils results in further stabilization of the molecule [16].

In addition to hydrophobic interactions, coiled-coil structure is also characterized by electrostatic interactions between residues in positions *e* and *g* of the heptade repeats. The rule of such bond formation is $i \rightarrow i' + 5$, i.e. the *g* residue of one helix forms an ion pair with residue *e* of the following heptade of another helix (usually, glutamate with lysine). Such interactions fulfill several important functions. First, they account for additional stabilization of coiled-coil structure. According to calculations by Hodges et al., formation of a single ion pair contributes approximately 1.5 kJ/mol to coiled-coil stability [17]. Second, formation of such bonds provides for specificity of coiled-coil structure formation. The nature of charged residues in positions *e* and *g* influences formation of homo- or heterodimers of α-helices as well as of coiled-coils with parallel or antiparallel subunit orientation [18]. Note that the above described interactions outline only general principles of coiled-coil structure organization. In each particular case, various specific interactions between amino acid residues contribute to coiled-coil stability, formation specificity, and the supercoil oligomeric status.

It is wrong to believe that the structure of all known proteins of this class corresponds to the canonical double helix (formulated as the PV-hypothesis). The literature describes numerous deviations from canonical structure, which often appear in coiled-coil proteins and are in one way or another important for their functioning. For example, segment 2B of the vimentin molecule (protein of intermediate filaments) forms a double helix characterized by interruption of the heptade structure by three additional residues that locally disturb periodicity of amino acid sequence (the so-called "stutter") [19]. Owing to this, there is a local unwinding of the double helix [10], which, as became clear, is important for correct assembly of vimentin monomers into intermediate filaments [20]. Besides, some proteins with coiled-coil structure are characterized by the presence of polar or charged residues in their hydrophobic core. For example, His106 residue of the double-helical domain of influenza virus hemagglutinin undergoes ionization at lower pH, which appears to be the basis for the conformational rearrangements of the molecule underlying the mechanism of virus penetration into a cell [21]. Yeast transcription factor GCN4 is characterized by coiled-coil structure with high content of leucine residues in the hydrophobic core ("leucine zipper"). Nevertheless, in one of its *a*-positions there is a conservative asparagine residue that, surprisingly, is responsible for preferable formation of dimeric rather than trimeric protein [18]. Thus, these examples show that the presence of noncanonical residues (i.e. contradicting the logic of coiled-coil formation) in the coiled-coil structure is often a necessary condition for proper functioning of a protein.

Factors that define stability of coiled-coil structure. Below we shall briefly outline the factors that provide for stability of coiled-coil proteins.

1) Since the coiled-coil structure is in essence a bundle of α-helices, its stability will be directly defined by the

stabilities of these helices. This parameter is known to depend directly on amino acid sequence because amino acids differ in the so-called *helical propensity*. As follows from experiments on measuring the energy of α-helix stabilization by different amino acids, the highest helical propensity is characteristic of methionine, alanine, leucine, glutamate (in uncharged form), and lysine. On the other hand, the lowest ability to form α-helices is characteristic of proline and glycine. Proline is an imino acid and causes a ~30% bend in the α-helix, while glycine exhibits a destabilizing effect due to its high conformational mobility and, as a result, to the effect of the entropy factor [22].

2) *Hydrophobicity of amino acid residues localized in the helical interface, and favorable electrostatic interaction* (considered above).

3) *Packing density of the hydrophobic core of the molecule.* Hodges introduced the notions of stabilizing and destabilizing clusters that designate the groups of amino acid residues stabilizing (Leu, Ile, Val, Met, Phe, Tyr) or destabilizing (Gly, Ala, Cys, Ser, Thr, Asn, Gln, His, Arg, Lys, Trp) the coiled-coil hydrophobic core (Fig. 1A). It was noted that in many natural coiled-coil structures (including TM and myosin rod) clusters of both types alternate with each other as well as with regions in which destabilizing residues alternate with stabilizing ones [23]. Experimental data [24] show that such regions significantly destabilize coiled-coil structure, mainly due to decreased density of hydrophobic core packing and its exposure to the solvent molecules.

Tropomyosin Isoforms and Their Distribution

The presence of TM is noted both in muscle tissue and in non-muscle cells (platelets, brain cells, fibroblasts) [25]. Also, two TM species were found in yeast cells [26]. The Human Genome Project and the achievements of functional genomics and molecular cloning revealed the existence of four human TM genes: *TPM1* (formerly called α-Tm), *TPM2* (β-Tm), *TPM3* (γ-Tm, hTm30nm, or hTmnm), and *TPM4* (δ-Tm, hTm30pl, or hTmpl). The following chromosomal localization of these genes was registered: *TPM1* – 15q22.1, *TPM2* – 9p13.2-p13.1, *TPM3* – 1q21.2, *TPM4* – 19p13.1. Alternative splicing, the use of alternative promoters, and polyadenylation sites provide for the presence in the human transcriptome of at least 22 various mRNA containing full-sized TM open reading frames, which was confirmed by direct cloning from human cells and tissues [27]. Historically, tropomyosins have been divided into two groups based on the number of amino acid residues: 1) TM of high molecular weight (HMW) containing from 281 to 284 amino acid residues; 2) TM with low molecular weight (LMW) containing 247 amino acids in their primary structure. Virtually all muscle TMs belong to the HMW group, whereas most non-muscle TMs belong to the LMW group. Such difference in size is the result of use of alternative promoters upon transcription of the TPM genes: external promoter is used in the case of HMW, while internal promoter is used in the case of LMW [28].

Taking into account the fact that TM was initially isolated and characterized as a muscle protein, we shall consider the isoform composition of TM from muscle. In mammalian skeletal muscle transcripts of all four TM isoforms and corresponding protein products were identified [29]. Expression of two transcripts, α-Tm_{fast} (encoded by *TPM1* gene) and α-Tm_{slow} (encoded by *TPM3* gene) is detected in fast and slow muscle fibers, respectively. Two other transcripts, β-Tm (the *TPM2* gene product) and Tm4 (the *TPM4* gene product) are expressed in both fiber types. Proteins α-Tm_{fast}, α-Tm_{slow}, and β-Tm were identified within skeletal muscle, whereas the Tm4 protein product was not found. Observations show that β-Tm is mainly expressed in oxidative fibers; thus, this isoform can be found in type 1 fibers (slow, oxidative) and 2A fibers (fast, oxidative), but not in type 2B fibers (fast, glycolytic). Isoform α-Tm_{fast} is the main and often unique TM of cardiac muscle, although there are data showing that the β-Tm content in the heart paradoxically depends on the animal size: practically no β-Tm is found in mouse heart, whereas in human heart the β-Tm/α-Tm_{fast} ratio is ~1/5 [25, 30]. Data in the literature concerning the quantitative isoform ratio are quite contradictory. It is assumed that in skeletal muscle the ratio of all α-isoforms and β-Tm is (3-4) : 1 [25]. Thus, the existence of three types of TM dimers – αα, αβ, and ββ is possible. In this case, TM of skeletal muscle *in vivo* is 90% represented by the first two types (αα and αβ) [25]. It has been shown recently that some mutations in the *TPM2* gene, resulting in motor disorders, influence quantitative composition of TM isoforms in skeletal muscle [31]. In this case, increased level of β-Tm expression and prevalent formation of ββ-homodimers instead of αβ-heterodimers is observed, which, according to the authors' opinion, is followed by pathological consequences. The two types of tropomyosin chains, α and β, differ by only 39 amino acid residues (~14%) and the α-chain contains just one cysteine residue (Cys190), while the β-chain contains two residues (Cys36 and Cys190).

The main TM isoforms in smooth muscle are Tm6 (smooth muscle isoform of α-TM, the *TPM1* gene product) and Tm1 (smooth muscle isoform of β-TM, the *TPM2* gene product) [27], and in this case each TM chain contains a single cysteine residue, Cys190 in α-chain and Cys36 in β-chain. Unlike skeletal muscle, in smooth muscle (e.g. in chicken gizzard) TM is almost exclusively represented by heterodimers α-Tm/β-Tm [32].

Specific Features of Tropomyosin Primary Structure

As already mentioned, the molecule of the best-studied TM (α-TM of skeletal muscle) consists of two

chains, each including 284 amino acid residues. This corresponds to 40 full heptads. The most striking feature of the TM molecule is that its heptade sequence is never interrupted (does not form the above-described "stutter") with the only remarkable exception of tropomyosins isolated from baker's yeasts *Saccharomyces cerevisiae*. In the latter case, there are "pauses" in the TM molecule, i.e. regions of disturbed heptade periodicity [33], which are absent from all other studied TM proteins. There are numerous examples in the literature dealing with analysis of TM primary structure. The author K. Holmes writes in one of his articles that "the periodic features of tropomyosin are a numerologist's dream" [34]. Below we shall mention the periodicity related to actin-binding properties of TM. Now we shall only note that TM, like different proteins with coiled-coil structure, is characterized by the presence of stabilizing and destabilizing clusters in a hydrophobic core (see above). According to Hodges, the α-TM molecule of skeletal muscle contains nine stabilizing and six destabilizing clusters [23]. There are also regions in which alternation of stabilizing and destabilizing amino acids is observed (for example, residues 100-190, i.e. the central part of TM molecule). Thus, these regions should be characterized by low compactness of the double helix hydrophobic core and resulting low structural stability [24]. Another interesting observation is that the most widespread destabilizing residue in the TM molecule is alanine. Taking into account that Ala has very high helix-forming ability, one can conclude that evolution of the TM structure was directed towards local destabilization of the coiled-coil double helix with retention of stability of α-helical structure on the whole.

Stability of the Tropomyosin Molecule

The coiled-coil structure is unique because, unlike other structures, its stability can be predicted on the basis of the amino acid sequence of the protein. Certainly, it is also interesting to follow such relationship in the case of TM, because numerous works dealt with investigation of its stability (mainly thermal stability).

The arsenal of experimental approaches to investigation of TM is rather broad, which is explained by the structural peculiarities of the protein. Since the coiled-coil is an α-helical structure, registration of temperature dependences of circular dichroism (CD) is, obviously, a relevant approach for investigation of TM thermal stability. As is known, the CD spectra of α-helical structures are characterized by a pronounced negative extremum at $\lambda = 222$ nm. Correspondingly, CD temperature dependences at this wavelength reflect the loss of α-helical structure as temperature increases. Using this approach, Lehrer showed that the heat-induced denaturation of TM is reversible, and temperature dependence of CD for α-TM from rabbit skeletal muscle is characterized by a sharp loss of helicity observed in the range of 40-50°C [35]. The dependence of the half-transition temperature on protein concentration indicated that TM denaturation is accompanied by reversible dissociation of its polypeptide chains. Later this was confirmed by Holtzer et al. who studied temperature dependences of light scattering of α-TM from rabbit skeletal muscle [36]. It was also shown that this main transition is preceded on the temperature dependence curves by a small "pre-transition", the nature of which remained unclear [37]. Thus it was supposed that TM melting is not a simple one-step reversible process, but that it is accompanied by formation of a partially unfolded intermediate. In addition, it became clear that the presence of so-called "pre-transition" depends on the redox state of SH-groups of the Cys190 residue in the skeletal muscle α-TM, since it only appeared upon disulfide bond formation between the TM chains [35]. These data indicated that the disulfide bond causes conformational constraints and following destabilization of the TM molecule. Another important achievement was identification of the molecule region corresponding to the "pre-transition". By means of chemical formation of disulfide bonds, it was shown [6] that at temperatures preceding the main transition a local unfolding of TM in the region of Cys190 occurs [35]. Thus, the term "local unfolding" was introduced for the first time, and the lowered stability of the central part of the TM molecule was suggested. When works on CD are considered as applied to TM, it is also necessary to mention investigations by Greenfield and Hitchkock-DeGregori, who made fundamental contributions to the study of TM structure and stability. In particular, they showed that thermal unfolding of α-TM from skeletal muscle is not a fully cooperative process. Using various approaches to CD spectra deconvolution, they showed that upon heating from 0 to 25°C the contribution of coiled-coil structure into the CD spectrum decreases, whereas helicity increases, and the contribution of disordered structure does not change. As expected, during the main transition portions of both coiled-coil structure and α-helix decrease, while the contribution of disordered structure of course increases [38].

Another important approach to studying the stability and conformational properties of TM is a method based on fluorescence of pyrene excimers. Usually there is a unique cysteine residue in each TM chain, Cys190 in the case of α-TM of skeletal and smooth muscle, or Cys36 in the case of β-TM of smooth muscle (skeletal muscle β-TM is an exception, as it contains both Cys190 and Cys36). This makes possible specific modifications of the protein by fluorescent dyes, in particular by pyrene (usually, either pyrenyl-iodoacetamide or pyrenyl-maleimide). A special property of pyrene molecules is the ability to interact with each other, thus forming excited dimers, or excimers. Excimers are characterized by a

longer-wavelength maximum of the fluorescence peak compared to the monomeric pyrene form [39]. Correspondingly, conformational changes in the region of pyrene-modified cysteine residue will influence the quantum yield of excimer fluorescence. This approach revealed several important facts. First, spatial proximity of Cys190 residues belonging to different TM chains was confirmed [39]. Second, it was shown in the same work that TM melting is accompanied by complete quenching of excimer fluorescence, which confirms the dissociative mechanism of TM denaturation. Third, it was shown that pyrene label attached to TM exists in two equilibrium conformations, conformation A characterized by low yield of excimer fluorescence, and conformation B in which excimer formation is more favorable [40]. Conformation B is characterized by a local separation of TM chains in the label region, which explains the high yield of excimer fluorescence. The curve of temperature dependence of pyrenyl-labeled TM excimer fluorescence is characterized by increase and then a sharp decrease. This shape of the curve is easy to explain by redistribution of pyrene labels between the A and B populations in a temperature-dependent regime [40]. Thus, the use of pyrene labels confirmed the previous assumption that TM thermal denaturation proceeds with formation of an intermediate characterized by local chain separation in the region of Cys190. This means that TM denaturation follows a more complex mechanism than a reversible one-step transition and can be described by the following scheme: $NN \leftrightarrow XX \leftrightarrow 2D$, in which *NN* corresponds to the native state of TM dimer with pyrene in A conformation, intermediate *XX* state with the pyrene predominantly in B conformation, while *2D* state corresponds to two separate TM chains after dissociation of the dimer [41]. The use of pyrenyl-labeled TM later played an important role in studies on its regulatory properties.

A quite useful tool for investigation of TM stability and conformational properties is limited proteolysis. The earliest works on TM cleavage by trypsin can be traced back to 1960-1970. Already at that time Ooi [42] as well as Gorecka and Drabikowski [43] showed that incubation of TM with trypsin is accompanied by formation of fragments with molecular masses of 17, 19, and 12 kDa. Later Smillie et al. showed that the initial TM trypsinolysis takes place between the Arg133 and Ala134 residues [44]. Having a coiled-coil structure, TM is characterized by a high content of charged residues (as a rule, in positions *e* and *g*). This explains the presence in TM of a large number of potential sites of trypsinolysis that are evenly distributed along molecule, since trypsin is active towards peptide bond between arginine and any other residue (except proline). Thus, trypsinolysis can be used to study local stability of the TM molecule. Since initial TM trypsinolysis only occurs at a single site (between Arg133 and Ala134), it was concluded that the central part of TM exhibits the lowest stability, i.e. the highest conformational mobility (flexibility) [45]. In 1984 Ueno carried out a detailed kinetic analysis of TM trypsinolysis. He found that the rate constant of trypsinolysis depends on such factors as temperature and the disulfide bond between Cys190 residues, introduction of which increased the rate of trypsinolysis at Arg133 [45]. Comparison of the results of trypsinolysis with CD data and pyrene excimers fluorescence showed that the above-mentioned intermediate of TM melting corresponding to the so-called pre-transition on curves of CD temperature dependence, is characterized by partially unfolded structure of the site including residues 130-190, i.e. to the middle part of the TM molecule. Thus, the concept of the TM central part as the less stable part of the molecule was formulated. Nevertheless, it remained unclear what was responsible for the low stability of this site.

In 2008 Lehrer et al. published a very interesting work directly connected with this question. They supposed that instability of the middle part of TM is accounted for by the presence of a noncanonical Asp137 residue in the *d* position of the heptade repeat [46]. Negative charge introduced by this residue into the hydrophobic core of the molecule in theory may cause electrostatic repulsion of α-helices and thus result in the destabilization of coiled-coil structure. The authors confirmed this hypothesis using mutant TM carrying the Asp137Leu mutation. It was shown that the mutant protein did not undergo trypsinolysis [46], which confirmed the involvement of Asp137 in destabilization of the central part of the TM molecule. Nevertheless, charged residues in the hydrophobic core of the TM molecule are also present in different regions of the molecule (for example, Glu218), but no cleavage is observed there despite the existence of potential sites of trypsinolysis. This shows that along with the presence of noncanonical Asp137 residue, some other factors, unknown until very recently, play an essential role in destabilization of the central part of the TM molecule.

Quite recently we managed to introduce some clearness into this problem. Careful analysis of the TM amino acid sequence made it possible to identify a conservative destabilizing glycine residue Gly126 in the middle part of the molecule in position *g* of a heptade repeat. The residue appears to be conserved in virtually all TM isoforms in many animal species. Replacement of this noncanonical glycine residue by canonical alanine or arginine caused the same effect as the Asp137Leu mutation, i.e. it completely prevented TM trypsinolysis at Arg133 [47]. Such a similarity of results shows that noncanonical residues Asp137 and Gly126 act in concert and destabilize the middle part of the TM molecule. Evidently, such a destabilization, necessary for cleavage at Arg133, is achieved only due to the concerted action of the Asp137 and Gly126 residues, because replacement of each of them by stabilizing residues results in inhibition of proteolysis. It seems quite probable that Gly126 only destabilizes individual α-helices, i.e. those that are not stabi-

lized by interaction with each other. Thus, noncanonical Gly126 most likely causes local unfolding of α-helices that separate in this region due to the effect of noncanonical Asp137, which makes these helices accessible to trypsinolysis between Arg133 and Ala134 (local divergence of TM α-helices in the region Asp137 was previously revealed during study of atomic structures of TM fragments [48, 49]). In this connection, it becomes clear why no trypsinolysis takes place in the region of different noncanonical glycine residues Gly52 and Gly188, although the TM molecule contains over 30 potential sites for such cleavage evenly distributed along the entire molecule: in the region of these glycine residues there are no charged/polar residues within hydrophobic core that could cause local separation of the α-helices. On the other hand, according to crystallographic data, residues Glu218 and Gln263 cause local separation of the TM α-helices, but nevertheless no trypsinolysis is observed in these regions due to the absence there of residues destabilizing α-helix.

Therefore, studying TM thermal denaturation by CD and fluorescent labels as well as the use of limited proteolysis showed that the TM molecule is characterized by heterogeneous stability, i.e. it contains both more and less stable regions. Further progress in this direction was achieved using the combination of CD, fluorescence spectroscopy, and mutagenesis as well as to application of differential scanning calorimetry (DSC). Pato, Mak, and Smillie used CD to study thermal stability of various fragments of rabbit skeletal α-TM and concluded that C-terminal fragments (tryptic fragment 134-284 and cyanogen bromide fragment 142-281) are characterized by lower stability (T_m ~ 34-37°C), whereas N-terminal fragments (tryptic fragment 1-133 and cyanogen bromide fragment 11-127) exhibit higher thermal stability (T_m ~ 47-50°C) [44]. Williams and Swenson studied skeletal muscle α-TM by DSC and showed that the shape of the excess heat absorption curve depended on the state of sulfhydryl groups of the Cys190 residue. In the case of complete reduction or modification of the SH group, the DSC curve was characterized by the presence of two peaks with temperature maxima T_m = 41.5°C and T_m = 52.5°C. Formation of a disulfide bond between Cys190 residues resulted in appearance of a main peak with T_m = 52°C and of minor low-temperature peak with T_m = 32°C on the DSC curve [50]. In addition, the authors studied DSC profiles of TM cyanogen bromide fragments (11-127 and 142-281) and were able to identify these thermal transitions, i.e. to reveal their correspondence to melting of separate regions of the molecule (calorimetric domains). The lower thermal stability peak on the DSC curve of reduced TM was attributed to denaturation of the C-terminal fragment, whereas the peak of the higher thermal stability was assigned to denaturation the N-terminal fragment [50]. This agreed well with almost simultaneously published and above-cited work by Pato et al. [44]. Thus, introduction of a disulfide bond into the TM molecule increases thermal stability of the C-terminal fragment (domain). Lehrer et al. analyzed these data and supposed that in this case the reason for stabilization is decrease in denaturation entropy (ΔS) of the TM C-terminal domain, because introduction of a cross-link decreases the amount of different denatured state conformations. As a result, free energy of denaturation transition (ΔG) grows and thermal stability of C-terminal domain increases [51]. Thus, disulfide bond formation between Cys190 residues of two TM chains exhibits a paradoxical effect: on one hand, increase in thermal stability of the TM molecule C-terminal domain occurs, while on the other hand, and simultaneously an increase in local melting "expressiveness" ("pre-transition") occurs. In the later literature there are also different works on investigation on thermal stability of various TM isoforms using DSC. The main result of these works is, for the most part, the confirmation of the presence of cooperative, reversibly denaturing blocks (calorimetric domains) in the TM molecule [52-55]. In more detail, results of the DSC studies on TM thermal denaturation were published by one of the authors in 2004 [56, 57].

It should be noted that the DSC technique has been already for over 10 years successfully used by our research team for studying special features of TM thermal unfolding. Thus, interesting results were obtained in 2006 during investigation by this method of non-muscle TM isoforms. It was shown for the first time that enthalpy of thermal denaturation of these TM isoforms is much less than that of muscle TM isoforms. These data indicated that some parts of the non-muscle TM molecules are melted non-cooperatively compared to thermal transitions characteristic of N- and C-terminal regions [58]. However, the most surprising is that the non-cooperative thermal unfolding (i.e. melting without expressed cooperative transition seen only in a gentle slope of the heat sorption curve) was registered even in such best-studied TM preparation as skeletal muscle α-TM [47]. In this case, replacements of the noncanonical Gly126 residue in the central part of the α-TM molecule by canonical Ala and Arg residues stabilized this region of the molecule, as could be judged by the change of the character of thermal unfolding of the α-TM central part from non-cooperative to cooperative. Moreover, special calorimetric approaches allowed us to calculate enthalpy of non-cooperative denaturation of the central part of the α-TM molecule: it was about 300 kJ/mol, which corresponds to about 60-70 amino acid residues, i.e. to one fourth of the molecule [47]. Note in addition that DSC is successfully used in our research group for investigation of TM thermal denaturation directly on the surface of actin filaments [47, 55-60], as well as in investigation of TM preparations carrying various myopathy mutations [55, 61-63]; these directions in investigations will be considered in some more detail in the following parts of this review.

In conclusion of this part we shall repeat key points concerning the structure and stability of the TM molecule.

1) The amino acid sequence of the TM molecule is indicative of its structural heterogeneity, i.e. the existence in the molecule of regions of different stability.

2) Experimental data confirm that different regions of the TM molecule are characterized by different stability (thermal stability).

3) In general, the TM molecule seems to be composed of two rather stable domains (C-terminal and N-terminal) joined by a linker – the molecule central part, exhibiting the lowest stability.

So, the arrangement of the TM molecule is far from the structure that could be expected from such simple structure as double α-helix. Thus, it seems necessary to discuss available atomic structures of the TM molecule in order to understand its structure and the relationship between structure and function.

Atomic Structures of Tropomyosins

Now data on the atomic structure of several fragments of skeletal muscle α-TM molecule are available, whereas the structure of the whole molecule is still not resolved. The main difficulty in the solution of this problem is obtaining high quality crystals. Despite the ability of TM to form easily the so-called para-crystals ("Bailey crystals" after the name of their discoverer), they are characterized by blurred X-ray diffraction pattern due to a high solvent content (>90%). Such crystals were used for obtaining information about the general form and package of individual molecules in the crystal at 7 Å resolution [64]. An important conclusion from this work was that the TM molecule exhibits high conformational mobility, causing a variety of crystalline and paracrystalline TM forms. Besides, variability of the superhelix pitch along the whole molecule and the presence of bends in the latter were noted (Fig. 2a) [65].

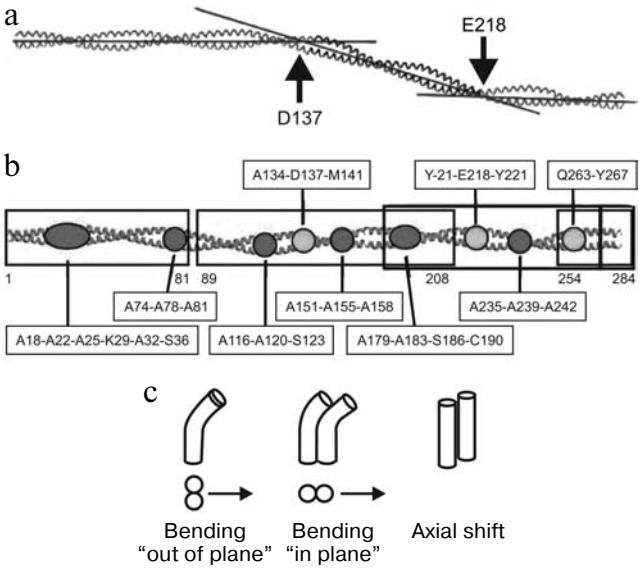

Fig. 2. Structural features of the TM molecule according to X-ray data. a) Structural model of TM at 7 Å resolution. Positions of residues D137 and E218 are shown with associated bending [65]. b) TM segments whose atomic structures are now available (in black frames). Dark circles designate the presence of alanine clusters in the hydrophobic core (cluster composition is shown in frames under the scheme), gray circles designate the presence of charged residues in it (shown in frames above the scheme) [67]. c) Structural features of the TM molecule [65] (in more detail in the text).

Structures of higher resolution were obtained for separate fragments of the α-TM molecule: the N-terminal fragment of chicken skeletal muscle α-TM (81 amino acid residues, 2.0 Å resolution) [48], central part of rat skeletal muscle α-TM (residues 89-208, resolution 2.3 Å) [49], C-terminal fragment of rat skeletal muscle α-TM (residues 253-284, resolution 2.7 Å) [66], and C-terminal fragment of rabbit skeletal muscle α-TM (residues 176-284, resolution 2.6 Å; residues 176-273, resolution 1.8-2.0 Å) [65, 67]. Let us consider main structural features of the TM molecule revealed by the available atomic models.

The structure for the N-terminal fragment of chicken skeletal muscle α-TM was the first to be resolved (PDB 1IC2). This fragment contains two alanine clusters in the hydrophobic core (Fig. 2b). A characteristic feature of the atomic structure of this N-terminal fragment is shortening of the distance between two TM chains in the region of residues 15-36 (the first alanine cluster) to 8 Å, whereas on the whole the interchain distance is 9.5-10 Å. Another interesting feature of the atomic structure of the N-terminal fragment is the axial shift of the TM chains relative each other by ~1 Å (Fig. 2c) that falls just within the region coinciding with the alanine cluster. The consequences of this shift are quite interesting; they are expressed in the appearance of clear bending of the TM molecule in the place of the noncanonical coiled-coil region (region of alanine cluster) as it transitions to the canonical one, i.e. that containing tightly packed residues in the hydrophobic core [48]. On average, the bending is by 6°. It was noted that the existence of such bends is a unique feature of TM, because nothing of the kind was observed for all other proteins with coiled-coil structure studied by that time by X-ray analysis. The authors supposed that such bends confer to the TM molecule a definite shape corresponding to the geometry of the actin filament surface and thus provide for productive actin binding. This hypothesis made it possible to explain the abnormally high content of alanine residues in TM, because this hypothesis suggests that, first, they stabilize the TM α-helical structure and, second, they form groups that are responsible for the specific shape of the whole molecule. As we have to make sure, this hypothesis was largely developed in subsequent works.

In 2005 the atomic structure of the central part of the rat α-TM molecule (residues 89-208; PDB 2B9C) was solved [49]. Note that this part of the molecule was of special interest for researchers because already in previous works significant instability of this region was noted. The X-ray analysis confirmed previously noted specific features of TM structure and in addition it revealed some new features. The authors noted the presence of alanine clusters (such as in the region of residues 150-160; Fig. 2b) and associated structural features (local shortening of the double helix radius, axial shift of chains, specific bending of the molecule) (see Fig. 2c). However, in addition to alanine residues collected in groups, the presence of alanines (Ala102, Ala109, and Ala116 in *d* positions and Ala134 in position *a*), alternating with canonical residues having a large hydrophobic radical (e.g. Met127 and Met141) was also noted. Such alternations of residues with different size side chains result in emergence of cavities in the hydrophobic core of the central part of the TM molecule, i.e. loose packing appears there. Detailed analysis of this structure showed that the existence of cavities in the core correlates with molecule bending, and in this case its level is directly associated with the volume of the cavities. The authors also found that this bending somewhat differs in shape from those in the region of alanine clusters. Although in the latter case both polypeptide chains are localized in the bending plane ("in plane" in Fig. 2c), in the case of bends caused by the presence of cavities in the core the TM chains are bent in different, though parallel, planes ("out of plane" in Fig. 2c).

Structure of the C-terminal fragment of rabbit skeletal muscle α-TM (residues 176-284, resolution 2.6 Å; residues 176-273, resolution 1.8-2.0 Å) was obtained in Maeda's group [65, 67]. The structures confirmed the existence of earlier described structural features (double helix narrowing and axial shift in the region of alanine clusters) and revealed formerly unknown new structural features of the TM molecule. Among them, most striking are interruptions of the hydrophobic core integrity expressed in the presence of cavities in three regions of the C-terminal fragment. The first (residues 207-214) and second (residues 214-221) cavities are so close to each other that they can be considered as two segments of the same cavity. Rather pronounced separation (~11 Å) of the two TM polypeptide chains associated with this cavity is noted along with local untwisting of coiled-coil, which is revealed in increased pitch of left-handed superhelix to 242-258 Å (the normal value is 156 Å). The hydrophobic core sequence in the region of the first and second cavities looks like Leu207(*d*)-Ala211(*a*)-Tyr214(*d*)-Glu218(*a*)-Tyr221(*d*). Among the above-mentioned amino acids it is easy to notice the charged residue Glu218 to which the leading role is attributed in formation of both cavities in the hydrophobic core of TM molecule. Detailed study of atomic structures revealed the presence in the described cavities of several water molecules (their amount depends on the crystal) establishing hydrogen bonds with residues Glu210 and Glu218. The third cavity is formed between residues Gln263(*d*) and Tyr267(*a*) of the TM hydrophobic core and coincides with divergence of TM chains involved in formation of so-called "terminal interactions" with the N-terminus of next TM molecule (see below in detail). Besides, in this (third) cavity there is also a water molecule establishing hydrogen bonds with hydrophilic residue Gln263(*d*). Evidently, interruption of the tightly packed hydrophobic core as well as the presence in it of water molecules should significantly destabilize the coiled-coil structure. From the physicochemical point of view, this is explained by lowering entropy due to water molecule structuring both in the hydrophobic core and in its immediate environment. The local increase of conformational mobility associated with the described regions of hydrophobic core interruption follows from increased values of temperature B-factor: in the region of Glu218 its value is 110.2 Å^2, while in the rest of the molecule regions the mean value of B-factor is 40-50 Å^2.

It should be remembered that interruption of the hydrophobic core of the TM molecule also takes place in the region of charged residue Asp137(*d*). However, lower resolution of the central TM fragment structure and high value of B-factor associated with Asp137(*d*) prevented the localization of a water molecule in this region. Nevertheless, there is no doubt that all regions of the TM hydrophobic core, containing charged or neutral hydrophilic residues, exhibit low stability and high conformational mobility. That evolutionary conservatism of such regions, for example, residue Glu218, exists both in *Caenorhabditis elegans* and in *Homo sapiens*, is indicative of their functional importance. Meanwhile, now the functional significance of destabilization associated only with the third cavity in C-terminal fragment of the TM molecule is known. Hitchcock-DeGregori et al. showed that local destabilization caused by the Gln263(*d*) residue is necessary for successful interaction of the C-terminal region of a TM molecule with the N-terminus of another TM molecule as well as with the C-terminal segment ("tail") of troponin T [68]. That destabilization of the central part of the TM molecule caused by the presence of the Asp137(*d*) residue was confirmed by Lehrer et al. [46], but they did not disclose its functional significance. There is still no data in the literature concerning the functional importance of Glu218 and the destabilization it causes.

In addition to above-described breaks in hydrophobic core, the authors of the atomic structure of the TM molecule C-terminal fragment described another interesting observation. Since researchers managed to obtain different types of crystals, it became possible to compare TM structures obtained from different crystals. It was noted that such coiled-coil parameters as the double superhelix diameter and its pitch are independent of crystal type [65, 67]. The presence and size of bends in the

molecule, on the contrary, strongly depended on crystallization conditions and individual molecule packing in lattice points. The authors supposed that such behavior is indicative of high conformational mobility of the TM molecule, because this might explain the susceptibility of the molecule to deformations during crystallization. Brown [69] made detailed analysis of the TM molecule bends on the basis of information concerning all known crystalline structures of different TM fragments. Results of statistical data processing showed that the direction of the TM molecule bends is conservative in all studied crystalline structures and depends mainly on the protein primary structure. The bending level, in contrast, is completely defined by the environment of the molecule in the cells of the crystalline structure. Thus, the angle of bending is defined by external factors, independent of TM primary structure. However, if a bend is still formed, its direction is defined only by "internal" factors, i.e. by the amino acid sequence of the molecule.

Several conclusions can be drawn to complete the analysis of known atomic structures of TM. First, structure of the TM coiled-coil varies in different regions of the molecule: regions corresponding to destabilizing clusters differ in structural parameters from all the rest regions. Second, the TM molecule is a rod with high conformational mobility revealed in appearance of bends in some regions in response to external factors. Third, X-ray crystallography does not produce information able to explain the low stability of the central part of TM compared to other parts of the molecule, as well as difference in stability of the N- and C-terminal fragments of TM.

FUNCTIONAL PROPERTIES OF TROPOMYOSIN

At the very beginning of our analysis of the functional properties of TM, we shall outline the circle of problems to be discussed. First, TM is an actin-binding protein, and therefore we shall try to focus our attention on consideration of just this aspect. Since all the other functional properties of TM are secondary relative to interaction with actin filaments, we shall limit our consideration to a single such function of TM, namely the regulation of ATP-dependent interaction of myosin with actin, because just this process is in the basis of the regulation of muscle contraction.

Interaction of Tropomyosin with Actin Filaments

The question concerning TM binding to F-actin has been central during the whole history of TM investigation. Already in the 1960s it was noted that actin preparations obtained at that time contained up to 30% TM (cited by [25]). Ultracentrifugation of such contaminated actin preparations showed that TM migrated together with polymeric actin (F-actin) only at a definite ionic strength. It was also shown that TM does not interact with monomeric G-actin. The coprecipitation approach made it possible to determine that the stoichiometry of TM and F-actin complexes corresponds to their molar ratio 1 : 7 [70]. It was also shown that at low ionic strength (20 mM KCl and 1 mM $MgCl_2$) no TM binding to actin is observed (cited by [25]). Optimal conditions for binding were defined as 100 mM KCl and 5 mM $MgCl_2$, whereas at higher ionic strength the binding of TM to F-actin became noticeably weaker. At the same time, it was first shown that binding parameters (optimal ionic strength) are different for proteins from different sources [71].

Factors defining cooperativity of actin–tropomyosin interaction. Eisenberg et al. used Scatchard's technique and were among the first who showed that TM binding to fibrillar actin is a highly cooperative process [72]. In historical aspect, it is interesting that even earlier Drabikowski et al. used an analogous method and drew a conclusion about the existence on actin of regions with high and low affinity to TM. Nevertheless, later these results were recognized as an artifact (cited by [72]). Eisenberg et al. showed the existence of positive cooperativity in the interaction of actin with TM, but at that time they could not explain the nature of this fact. Since the affinity of the first few TM molecules to actin was significantly below that of following molecules, the authors supposed that interactions between TM molecule termini can be main factors responsible for cooperativity [72]. Another interesting observation of these authors was that actin filaments treated with glutaraldehyde, forming intra- and intermolecular cross-links, lost their ability to bind TM. This was an indirect indication that conformational changes in the actin filament might be a part of the positive cooperative of binding of TM to actin.

Importance of the TM termini interactions with formation of polymers for functioning of TM has been confirmed in numerous works. In particular, it was shown that cleavage using carboxypeptidase A of 11 amino acid residues [73] or four residues [74] from the C-terminus of the molecule results in the loss of the ability of TM to polymerize and bind actin filaments. Enzymic removal of the N-terminal peptide using bacterial peptidase OmpT gives similar results [75]. Hitchcock-DeGregori and Heald demonstrated the importance of acetylation of the N-terminal methionine residue of TM for actin binding [76]. They used recombinant TM expressed in *E. coli*, which explains the absence in it of N-terminal acetylation. In subsequent works several attempts were made to bypass this problem. In particular, it was shown that addition of the Ala-Ser dipeptide to the N-terminus of recombinant TM restores actin binding [77]. Note that this approach has now became most popular. In addition to imitation of acetylation using the Ala-Ser dipeptide, TM expression is possible in the baculovirus system (SF9 line of insect cells) [78, 79], in yeasts *Pichia pastoris* [80],

as well as TM coexpression with acetylating complex NatB in *E. coli* cells [81]. Nevertheless, imitation of acetylation remains the most available and an advantageous method for increasing the affinity of recombinant TM to F-actin.

Pronounced progress has been achieved over time in detection of the molecular architecture of the terminal contacts of TM. Omitting numerous details, we shall note work [82] in which the structure of the complex of the N- and C-terminal peptides of TM in solution was determined by NMR as well as work [83] in which crystal structure of the analogous complex was described. Both works describe divergence of polypeptide chains at the TM C-terminus and penetration of eleven N-terminal residues into the formed gap (Fig. 3a; see color insert).

Returning to cooperativity of the TM–F-actin interaction, we shall note that most probably not only terminal interactions between TM molecules specify its cooperative binding to actin filament. Tobacman, based on bioinformatics analysis of various TM isoform sequences and on experimental data, noted that cooperativity of TM binding to actin is independent of the size of overlapping TM regions involved in the terminal interaction [84]. Thus, in the case of yeast TM, the level of C- and N-termini overlap involves no more than five amino acid residues, but nevertheless, cooperativity of their binding to actin is not less than that in animal muscle TM. So a question arises concerning existence of different factors that along with terminal contacts between TM molecules provide for cooperativity of TM binding to actin. Most probably the actin filament proper can serve as such factor, and conformational changes in it can be a mediator in the cooperative process of TM binding to actin [84]. This hypothesis, despite its somewhat speculative character, is not completely senseless. There are rather many works in the literature showing that actin filament is not a passive structure. The well-known myosin effect (more exactly – effect of isolated myosin head or myosin subfragment 1, S1) on binding of TM to actin can serve as an example: under conditions of low affinity of actin to TM, the "strong binding" of S1 to the actin filament causes sharp increase in affinity of the latter to TM [85, 86]. Since direct interaction between S1 and TM has not been proved, it is logical suppose an effect of S1 under "strong binding" conditions on the conformational state of actin filaments, which finally results in a significant increase in the affinity of F-actin to TM.

Factors that define affinity of tropomyosin to F-actin. The fact that one TM molecule binds simultaneously seven actin monomers within a filament is indicative of the existence of at least seven actin-binding sites in the TM molecule. Analysis of the amino acid sequence of TM revealed seven equidistant clusters of acidic amino acid residues (Asp, Glu) in positions *b*, *c*, and *f* of heptade repeats, i.e. on the surface of the TM superhelix (cited by [25]) (Fig. 3b). McLachlan and Stewart [87] and later Phillips et al. [88] carried out similar analysis and determined the length of such actin-binding motif in the primary structure of TM (~39.3 residues) and revealed acidic residues that, in their opinion, must play a role in actin binding (Fig. 3b). They also divided each such motif into two segments called α- and β-zones, and supposed that these zones are involved in the contact of the TM molecule with actin in the different states of the thin filament (see below), thus causing the translocation of TM on the actin surface. The property in common for all such actin-binding motifs was their quasi-periodicity – though clusters of acidic residues repeated, they differed in composition in different regions of the molecule.

Hitchcock-DeGregori and her colleagues in many of their works investigated the properties of individual actin-binding motifs of TM (often called in the literature the actin-binding repeats) using mutagenesis. Their early works showed that the actin-binding function requires the presence of an integer (not fractional) number of actin-binding repeats in the TM molecule. The change of a single repeat length strongly decreased the actin-binding ability of the TM [89]. They showed in a similar way that specific structure of each repeat plays the defining role in the interaction of TM with actin, because replacement of any repeat by a site with disordered structure or by "leucine zipper" strongly decreases the affinity of TM to actin. Besides, it was shown that TM mutants with deletions of repeats 2, 3, 4, and 6 were altogether characterized by comparable affinity to actin, approximately 10-30 times weaker than in the wild-type TM. In contrast, the deletion of repeat 5 (and especially of its N-terminal part, residues 166-186) resulted in the loss of affinity of TM to actin both in the presence and in the absence of myosin head (S1) [89, 90-92]. Similarly, replacement of the amino acid sequence of the repeat by that of transcription factor GCN4, also having coiled-coil structure, had dramatic consequences only in the case of repeat 5 [92]. Repeat 5 has the most conservative sequence compared to other repeats: 31% of its sequence is identical in representatives of animals, whereas for the other repeats this parameter is only 17%. The N-terminal part of repeat 5 (residues 166-186) is characterized by even higher conservatism of 38%. The sequence of repeat 5 is encoded by exon 5 that never undergoes alternative splicing [25, 28]. However, what makes repeat 5 so unique from the point of view of the actin-binding function of TM? Hitchcock-DeGregori and Singh paid attention to the fact that alanine cluster Ala179-Ala183-Ser186, i.e. one of the above-mentioned destabilizing factors, is present in the structure of repeat 5. Such observation immediately generated a concept that just destabilization of repeat 5 structure may be rather significant for TM binding to actin. To check this hypothesis, they designed a mutant TM in which the alanine cluster sequence was replaced by more hydrophobic and therefore stabilizing residues (Ala179Leu-Ala183Val-Ser186Leu). The experiments

showed that these mutations really increased the thermal stability of TM, and studying temperature dependences of fluorescence of excimers of pyrenyl-iodoacetamide bound to Cys190 confirmed local stabilization of this region of the TM molecule. Replacement of a destabilizing cluster by a stabilizing one also caused a dramatic decrease of affinity of TM to F-actin [93]. Thus, it was shown that destabilization of coiled-coil structure in the region of repeat 5 due to the presence in hydrophobic core of noncanonical residues is a necessary condition for binding of TM to actin. In this work [93] the authors also demonstrate the necessity of the presence of conservative (mainly acidic) residues in positions *b*, *c*, and *f* of a coiled-coil heptade repeat. Remember, amino acid residues in these positions are localized on the surface of the TM double helix and can establish contacts with superficial residues of an actin filament.

Thus, authors assumed that the structure of TM should meet two requirements for actin binding: 1) conformational mobility ("flexibility") allowing the ligand (TM) molecule to adjust its structure to the geometry of the target molecule (fibrillar actin) surface; 2) interaction specificity, i.e. existence of regions establishing specific contacts in the ligand and target structure [94]. Note that this concept is evidently fully applicable only to actin-binding repeat 5. In the case of repeat 2, as shown by the same authors, destabilization of coiled-coil structure by an alanine cluster also defines TM binding to actin [95], but deletion of repeat 2 or its replacement by a leucine zipper has a weak effect on the affinity of TM to actin [89].

So, works by Hitchcock-DeGregori and Singh [93-95] showed what was already suspected long ago: conformational lability ("flexibility") of the TM molecule is important for its binding to actin. It should be noted that these works correspond quite well to the spirit of time, i.e. in parallel with them the concepts of the importance of conformational mobility of intrinsically disordered proteins for their interactions with partner proteins are formulated in the literature. Nevertheless, in 2008 Lehrer et al. published a work in which they showed that stabilization of the structure of repeat 4 had no effect on the affinity of TM to actin [46]. As discussed above, there is a considerable set of evidence that the central part of the TM molecule is its most unstable region. Due to this, it was logical to suppose that stabilization of this region will affect the affinity of TM to actin, but nothing of this kind was found. Note that the authors did not suggest explanations of this phenomenon. Moreover, a similar phenomenon was also found in our investigations: it was shown that stabilization of the central part of the TM molecule upon replacement of noncanonical residue Gly126 by canonical residues Ala and Arg had no effect on the affinity of TM to F-actin [47]. Thus, a question arises about a change of the approach to the problem. Is the TM molecule as mobile as we imagine, and how important is this conformational flexibility for the interaction of TM with an actin filament? These questions became the key ones in a number of works that used an absolutely different approach to the problem.

The Gestalt-binding hypothesis. It is not difficult to understand that the above-described model of Hitchcock-DeGregori resembles in first approximation the induced fit hypothesis known from enzymology. According to this hypothesis substrate binding causes conformational changes in the enzyme active center, which makes possible further progress of the reaction. Conformational flexibility of actin-binding sites, as postulated in the Hitchcock-DeGregori model, is a necessary condition for formation of correct bonds between TM and actin. Holmes and Lehman (mainly due to staff members of a restaurant in Heidelberg not interfering in discussions after closing hour [96]) proposed a quite different mechanism of actin binding to TM. The following facts were starting points in their arguments. First, as shown by measurements, a single TM molecule binds to actin very weakly and is characterized by $K_a \sim (2\text{-}5)\cdot 10^3\ M^{-1}$ [97]. If the affinity of all actin-binding repeats of TM molecule are identical, then the constant of a single repeat binding should be ~3, which corresponds to no more than one electrostatic interaction [96]. Thus, temperature fluctuations would easily cause dissociation of one TM molecule from the actin filament surface. Taking into account that such low affinity on the whole is not characteristic of actin-binding proteins, it seems that stereospecificity is not the main factor causing the interaction of TM with an actin filament [96]. Reconstruction results obtained during investigation of the diffraction pattern of ordered actin–tropomyosin gels showed that the TM molecule is located at a distance of ~39 Å from the actin filament axis [98]. Using the TM atomic coordinates in electron-microscopic reconstructions of regulated thin filaments gave similar results: 40 Å in the presence of calcium and 42 Å in a calcium-free system [99]. Evidently, such a long distance between two protein surfaces (Fig. 3c) excludes formation of stereospecific contacts, i.e. interaction between actin monomers and actin-binding TM repeats results in simple electrostatic attraction, but not in formation of specific ion pairs [96]. Holmes and Lehman postulated that the observed specificity of TM binding to actin is explained by complementarity (congruence) of the two protein surfaces rather than by formation of specific contacts. They called such interaction Gestalt binding. The term is dualistic because Gestalt in translation from German means "shape" of the TM molecule as a key factor in interaction with actin. But Gestalt is also related to gestalt theory originating in psychology. This theory postulates that the properties of a system cannot be merely the sum of properties of its components. Thus, the authors wanted to show that the phenomenon of TM binding to actin cannot be explained via focusing attention at a single TM molecule and corre-

sponding F-actin site, and they suggested considering the actin filament and actin-bound TM as a whole.

Holmes and Lehman do not confine themselves to compilation of speculative hypotheses and use experimental data as arguments. In particular, they state that available atomic structures of TM fragments [48, 49] can be easily installed into tropomyosin filament contours from the Lorenz–Holmes model [98] as well as into electron-microscopic 3D reconstructions of thin filament [99]. They especially stress the fact that it is unnecessary to use deformations or different manipulations with atomic structures of TM fragments to install them into available reconstructions of actin–TM complexes. In addition, Holmes and Lehman pay attention to coiled-coil bends that are present in the TM atomic structures as factors that define specific shape of the molecule. It is very interesting that they describe as arguments the Singh and Hitchcock-DeGregori data on mutagenesis of alanine clusters in TM [95] resulting in the loss of ability of TM to bind actin (note that the authors of this work interpret their results quite differently – see above). Based on these facts, Holmes and Lehman postulated the existence of three factors causing binding of TM to actin: 1) weak unspecific interactions between proteins; 2) specific TM shape complementary to the actin filament surface, and 3) terminal interactions between TM molecules. In the frame of this concept, TM binding to actin seems as follows (Fig. 4). First, single TM molecules randomly bind to actin with very low K_a (Fig. 4, a-c). This interaction is disadvantageous from the point of view of entropy factor, but complementarity of the TM molecule shape and actin filament surface helps in leveling entropic effects. It is especially emphasized that the increased conformational mobility of TM is not necessary in principle. Since one TM molecule (40 nm) corresponds to half F-actin helix turn, tropomyosin plasticity is not obligatory to "wind around" the actin filament. After reaching some critical concentration, separate TM molecules begin to appear on the actin filament surface and contact each other, which essentially increase their chances to remain on the actin filament surface (Fig. 4d). This process continues until full saturation of the actin filament (Fig. 4e) and, as can be noted, this has cooperative character. Thus, the binding of TM to an actin filament will be sufficiently firm, though at the local level such interaction is weak and unspecific [96]. To comment, we shall note that the model of Hitchcock-DeGregori et al. shows that TM binding to actin obeys universal laws of protein–protein interactions, whereas the Gestalt-binding points to a unique mechanism of interaction of TM with actin filament.

So, the mechanism proposed by Holmes and Lehman is an interesting model, but does everything in it agree with data from the literature? First, the model is based on data of X-ray analysis of TM molecule fragments pointing to the existence of certain molecule structure defined by specific bends [48, 49]. At the same time, crystallographic data of Maeda et al. [65, 67] as well as analysis by Brown [69] show that the existence of bends is not constant and can be an artifact of crystallization. Moreover, there is an opinion that the variability of bends is indicative of conformational mobility of TM. This agrees with classical works by Lehrer and Hitchcock-DeGregori also confirming the existence of high conformational flexibility in the TM molecule. Thus, the Gestalt binding model postulates that the TM molecule does not require extreme plasticity for actin binding, but nevertheless, the existence of such TM feature is, evidently, a proven fact. Therefore, it remains incomprehensible why evolution of the TM molecule structure followed the way of acquiring conformational mobility.

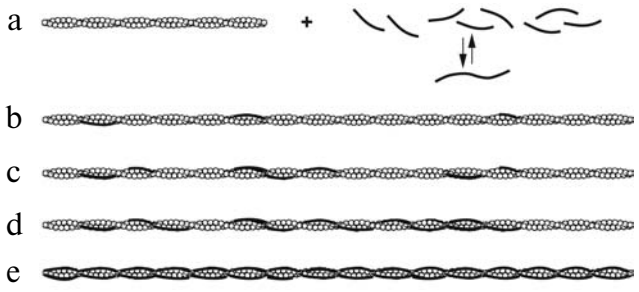

Fig. 4. Process of TM binding to F-actin (according to [96]). See explanations in the text.

It is impossible to ignore the works of Lehman's group investigating the mechanical properties of single TM molecules. These works followed the publication of the Gestalt binding hypothesis and were aimed at consideration of conformational mobility of the TM molecule. The most important among these works is paper [100] in which authors try for the first time to estimate quantitatively the conformational mobility of the TM molecule. Their methodology is based on calculation of the persistence length of TM from multiple electron microphotographs and molecular dynamic simulations. Let us consider this work in more detail. The authors used available atomic structures of TM fragments, which as already mentioned are sufficiently well adapted to the thin filament electron-microscopic reconstructions (Lorenz–Holmes model). Then TM molecules from this model were separated from actin, and molecular dynamic calculations were carried out, the main result of which was that the conformation of the TM molecule remained essentially unchanged: no local melting foci, chain separation, bending, etc. were formed. So the calculated apparent persistent length of the TM molecule was 104 nm, whereas the dynamic persistent length was five times longer (500 ± 40 nm). Such difference showed that the average conformation of the molecule differed from linear and is closer to an arch [100, 101]. The authors note that the value of the dynamic persistent length of TM (500 nm)

10-fold exceeds the similar parameter for the DNA molecule, and due to this the TM molecule acquires the name "semi-flexible" instead of "flexible". Using molecular dynamic calculations, the authors also showed that mutant TM with A74L-A78V-A81L substitutions, which lost the F-actin binding ability (the work by Singh and Hitchcock-DeGregori), is characterized by the intermediate shape of the molecule, different from the wild-type protein: mutation straightens the tropomyosin rod. The authors believe that this is a direct proof of validity of the Gestalt binding hypothesis.

Summarizing the analysis of works on mechanisms of interactions of actin with TM, we shall describe the main ideas that have appeared in the literature on this subject. There are two main hypotheses explaining the binding of TM to actin. One is based on conformational mobility of single TM molecules in solution and sees the process of interaction of F-actin with TM at the establishment of specific contacts stimulated by local instability of the actin-binding sites of TM. The second hypothesis rejects the importance of TM conformational mobility for the interaction with actin. Note that now there are significant experimental data in favor of each hypothesis, but at present there is still no way to combine or "reconcile" the two. The concept of the authors of this review is that both concepts are correct to some extent because each deals with different TM "life sides": on one side, TM in solution, and on the other side, actin-bound TM.

In conclusion of this section we shall consider a relatively recent work on the interaction of TM with F-actin. Lehman et al. used molecular modeling and calculated the equilibrium position of TM on the actin filament surface and identified amino acid residues that are most probably involved in the interaction of actin with TM [102]. They noted the long distance between α-carbon atoms of both protein polypeptide chains and relatively low specificity of electrostatic interactions between them, and they claim that the energetic landscape of various tropomyosin thread positions on the actin filament surface is relatively flat. Thus, different TM positions on F-actin surface are more or less equally probable. This is the main factor responsible for the involvement of TM in regulation of myosin–actin interaction, which is the basis of muscle contraction.

Thermal denaturation of tropomyosin within its complexes with F-actin. To finish consideration of works on the interaction of TM with actin, we would like to consider briefly how thermal unfolding of F-actin-bound TM proceeds. Changes in the flexibility of TM may correlate with changes in its thermal stability; therefore, investigations of the thermal denaturation of actin-bound TM using the DSC technique may give valuable information about the dynamic properties of TM on the surface of the actin filament. Due to this, DSC in combination with other methods is intensively used in our research group for investigation of thermal denaturation of TM complexed with F-actin. One of these methods is measuring temperature dependences of dissociation of TM–F-actin complexes, which are registered by changes in light scattering under the same conditions and at the same heating rate as in calorimetric experiments. In the first experiments using smooth muscle TM, the combination of these methods made it possible to show that TM thermal denaturation is accompanied by its dissociation from the F-actin surface [59]. Later this approach was successfully used for investigation of F-actin complexes with very different TM isoforms (α- and β-TM isoforms of skeletal and smooth muscle, "short" non-muscle TM α-isoforms, as well as yeast TM) [47, 55-58, 60, 61], and it was shown that the character of TM thermal denaturation changes significantly upon its binding to F-actin. This was registered by the appearance of a new highly cooperative thermal transition on the heat absorption curve of TM. After heating the TM–F-actin complex to 90°C and subsequent cooling (i.e. after complete irreversible denaturation of actin), this new peak completely disappeared, and during reheating, only peaks corresponding to thermal denaturation of free TM, whose melting is fully reversible, were observed on the DSC profile. This indicated that the appearance of a new peak in the presence of F-actin is indicative of thermal denaturation of actin-bound TM. Very good correlation was also found between the temperature maximum of this new actin-induced peak on the DSC thermogram and the temperature of dissociation of the TM–F-actin complex (i.e. the temperature at which 50% decrease of light scattering intensity of the complex was observed). This indicated that actin-induced changes in thermal denaturation of TM are caused by the dissociation of TM from the actin filament surface.

These results suggested the existence of the following mechanism of thermal denaturation of F-actin-bound TM [55-58]. Evidently F-actin protects bound TM against thermal denaturation, which is only possible upon dissociation of TM from the actin filament surface. Therefore, the new highly cooperative peak that appears on the DSC thermogram only in the presence of actin is indicative of thermal denaturation of those parts of the TM molecule that in the absence of actin should denature at temperatures lower than the dissociation temperature but could not do that in the presence of actin. These regions of TM denature in the presence of F-actin within a very narrow temperature range immediately after dissociation. All other more thermostable parts of the TM molecule, melted at a temperature higher than dissociation temperature, denature independently of actin after dissociation of TM from the surface of actin filament.

It should be noted that correct application of this effective approach developed in our research group has recently allowed us to calculate enthalpy of non-cooperative thermal denaturation of the central part of α-TM and so to estimate the size of this region of the molecule

(60-70 amino acid residues, i.e. approximately one fourth of the TM molecule length) [47].

Regulatory Functions of Tropomyosin

As already said above, all functions of TM are based on its ability to interact with actin filaments, and in one way or another they are connected with regulation of actin cytoskeleton. A fundamental property of TM is conferring flexibility and mechanical strength to the actin filament, which was shown for the first time using quasi-elastic laser light scattering [103]. TM also protects the actin filament against destabilization and fragmentation by such factors as DNase I, gelsolin, and cofilin, and it prevents filament branching mediated by Arp2/3 protein [104-107]. Thus TM is involved in the regulation of cell motility. To illustrate all this, we use results of investigation by Gupton et al. who showed that hyperexpression of exogenous TM dramatically alters dynamics of actin cytoskeleton and influences cell migration [108]. Cell lamellipodia usually contain a developed dynamic network of actin filaments along with high concentrations of cofilin and Arp2/3. At the same time, during movement into the cell actin cytoskeleton changes its own structure, and the repertoire of actin-binding proteins there emerges with more stable actin filaments containing TM and interacting with myosin II. Microinjections of TM into the leading cell edge decreases in it cofilin and Arp2/3 concentrations against a background of preserved cell motility. Nevertheless, although previously the migration mechanism was thought to be based on actin polymerization–depolymerization, it is now thought to be based on the work of molecular motors – myosin II heads that generate mechanical effort and movement during interaction with actin filaments.

Regulation of the interaction of myosin with actin filament is, perhaps, the most important functional property of TM, because it is directly related to the molecular mechanism of muscle contraction. Below we shall briefly consider present-day concepts of the muscle contraction mechanism, and we shall consider in detail regulation of this process by TM.

Mechanism of muscle contraction: general consideration. In a few words, the mechanism of muscle contraction called "sliding filament theory" is based on movement of two systems of protein filaments in parallel and toward each other. The issue is about thin (actin) and thick (myosin) filaments. Three key factors are responsible for this process. First, formation of "cross-bridges" – temporary contacts between thin and thick filaments [109]. Second, conformational changes occurring in the cross-bridges identified as globular structures ("heads") localized at the N-terminus of the myosin molecule [110]. Third, ATP hydrolysis by the myosin heads [110, 111]. Bagshow and Trentham used fluorescent spectroscopy and revealed a relationship between conformational changes of the myosin head (more exactly, myosin chymotryptic subfragment 1, S1) and the ATPase reaction cycle [112]. White and Taylor showed that removal of phosphate from the myosin head active center is the most important step of the ATPase reaction cycle and is coupled with force generation [113]. Several years earlier Lymn and Taylor showed that the ATPase activity of myosin is inhibited by reaction product (cleaved γ-phosphate of ATP) and that actin causes phosphate release, thus activating myosin ATPase [114]. Comparing their data with a hypothesis of Huxley concerning conformational changes of cross-bridges during muscle contraction, Lymn and Taylor suggested the following mechanochemical cycle (for the illustration of the well-known Lymn–Taylor scheme describing the working cycle of cross-bridges, see Fig. 5 in the paper by Koubassova and Tsaturyan published in this issue of *Biochemistry (Moscow)* [115]).

1. In the absence of nucleotide, the cross-bridge (myosin head) is strongly bound to actin filament and forms the so-called "rigor" complex (myosin "strong binding" to actin).

2. ATP binding to the myosin head active center causes rapid dissociation of the actomyosin complex. Then myosin hydrolyzes ATP and forms a stable enzyme–product complex M-ADP-P_i (where M is myosin head).

3. The M-ADP-P_i complex forms a weak interaction with actin filament.

4. The following strong binding with actin causes release of reaction products from the myosin active center, thus forming the initial strong (rigor) actin–myosin complex.

During the last step of the cycle in the cross-bridge a strong conformational change similar to an oar stroke occurs, which is responsible for force generation and provides for removal of ATPase reaction products from the myosin active center. Note that from the technical point of view it was unbelievably complicated to visualize the cross-bridge in motion. Elaboration of special approaches was necessary before creation of the first X-ray diagrams of the frog muscle with resolution in time, which demonstrated cross-bridge movements during contraction [116, 117].

Regulation of interaction of myosin head with actin filament. So, the interaction of actin with myosin, the basis of muscle contraction, follows the following principal scheme [118]:

$$\mathrm{A + M(N)} \xleftrightarrow{K_0} \underset{\text{collision complex}}{\mathrm{A \sim M(N)}} \xleftrightarrow{K_1} \underset{\text{A-state}}{\mathrm{AM(N)}} \xleftrightarrow{K_2} \underset{\text{R-state}}{\mathrm{AM(N)}},$$

where A is actin, M is myosin, N is nucleotide, A-state is the weak-binding state, and R is the strong (rigor)-binding state.

This scheme allows one to draw two important conclusions: 1) the interaction of myosin head with actin consists of two steps (if the quickly formed and short lived collision complex is not considered); 2) naturally, regulation of the interaction of myosin with actin can occur during one of these steps. Thus, it can be either regulation of interaction of myosin with binding sites on actin (the first and second equilibrium on the scheme), or regulation of the transition of myosin from the weak-binding state to the strong-binding state.

It was shown rather long ago that muscle contraction is launched upon increase of calcium ion concentration in the muscle cell cytosol in response to outer stimuli (innervation, chemical stimulation). In 1960 Ebashi et al. showed that Ca^{2+} binds a protein incorporated in thin (actin) filaments; they called this protein troponin (cited by [119]). It was shown that this protein is associated with one TM molecule and with seven actin monomers within a thin filament. Later it became clear that troponin is a complex of three proteins: troponin C capable of reversible binding Ca^{2+}, troponin I able to inhibit contraction independently, and troponin T that interacts with troponins I and C and tightly binds TM. The identification of troponin raised a new question: how can one troponin complex activate seven actin monomers within a thin filament? As an answer to this question the "steric blocking theory" was formulated on the basis of X-ray data. This theory postulates that in the absence of Ca^{2+} the TM molecule shields myosin-binding sites on an actin filament. When Ca^{2+} is bound to the troponin complex, TM is translocated on the actin filament surface, which makes these sites accessible for myosin head.

Let us consider some biochemical properties of reconstructed thin filaments. Isolated troponin C from skeletal muscle contains two sites that bind calcium ions cooperatively. The Hill coefficient of cooperativity in this case is estimated as 1.5-2.0 [120]. On the other hand, data on investigation of isometric force generation in response to increase in calcium concentration suggests more cooperative character of thin filament activation described by higher values of the Hill coefficient. This effect, i.e. inconsistency between the curve of calcium binding to troponin C and the curve of force generation (as function of $[Ca^{2+}]$) can be explained by the effect of TM – two calcium ions cooperatively activate one troponin complex that, in turn, activates not one but seven actin monomers, acting via TM. Results of further biochemical experiments showed that binding of myosin subfragment 1 (S1) to the TM-containing actin filament is cooperative, which is expressed in the sigmoid shape of the saturation curve [121]. Experiments of Lehrer and Morris, measuring actin-activated S1 ATPase as function of S1 concentration in the presence of TM, showed nonlinear dependence that is also indicative of cooperativity introduced by TM into the thin filament. In this case, at low S1 concentrations inhibition of its ATPase activity was observed, while at high S1 concentration, on the contrary, this activity increased [122]. The same effect is also registered in the system containing the fully reconstructed thin filament (F-actin–TM–troponin complex). It is difficult to explain these data if only two states of thin filament are considered – "OFF" (without calcium, myosin binding is impossible) and "ON" (in the presence of calcium, when myosin binding is possible). How can [S1] dependence of actin-activated S1 ATPase be nonlinear in the presence of calcium if in this case all binding sites of myosin head on actin are accessible? Evidently modification of a simple two-step scheme OFF ($-Ca^{2+}$) $\leftrightarrow$ ON ($+Ca^{2+}$) was required for resolution of all these contradictions. McKillop and Geeves studied binding of S1 to regulated thin filament using equilibrium titration and rapid kinetics techniques, and they concluded that for adequate description of the observed phenomena, the scheme of thin filament regulation should include three states rather than two [123]. They called these states **B** (***B**locked*, *no myosin head binding takes place*), **C** (***C**losed* = Ca-induced, myosin heads are capable of weak binding to actin filament), and **M** (***M**yosin-induced* or *Open*, myosin heads are able to form strong binding to actin) (Fig. 5).

Figure 5 graphically shows the essence of the proposed model. It is seen that the **C** $\leftrightarrow$ **M** equilibrium is defined by the presence of myosin in the system. Thus, introduction of this additional equilibrium explains the cooperativity of myosin binding to actin in the presence of TM. McKillop and Geeves supposed that the thin filament transition from state **C** to state **M** is coupled with the transition of myosin head from the weak binding state into strong binding [123]. Thus, they managed to combine in the frame of one scheme both regulatory processes and processes associated with the myosin ATPase reaction proper and force generation. It is necessary to note that the cooperativity effect suggests the existence of a cooperative unit: in our case it is the amount of actin monomers transferred to the **M** state by strong binding of a single myosin head. Let us consider in more detail the factors influencing different stages of the kinetic scheme of "three state theory" (Fig. 5b). Equilibrium constant K_T has value 0.2 in the presence of Ca^{2+} and value <0.2 without Ca^{2+}. Since this constant is defined by the properties of the thin filament itself, it is independent of nucleotide binding to S1, unlike constant K_2 equal to ~200 in the absence of bound nucleotides. Another important parameter influencing the **C** $\leftrightarrow$ **M** equilibrium is the size of the cooperative unit n. Since the ratio of cooperative units in **C** and **M** states is $\mathbf{M/C} = K_T(1 + K_2)^m$, where m is the number of actin monomers occupied by S1 within one cooperative unit [124], the size of the cooperative unit influences **M/C** (the higher n, the more chances that several S1 molecules will bind one cooperative unit, thus increasing the **M/C** ratio). Constants K_T and K_2 depend slightly on properties of the thin filament proteins, while the size of the cooperative unit exhibits strong depend-

ence on the properties of TM. For example, Lehrer et al. found that the size of the cooperative unit in the case of chicken gizzard smooth muscle TM was twice higher than in the case of TM from skeletal muscle [125]. The **B** ↔ **C** equilibrium depends on Ca^{2+} and is defined by equilibrium constant K_B. It was shown that these constant values are independent of TM isoforms, but they are strongly influenced by different troponin isoforms (cited by [126]).

Further investigations sought to elucidate structural features of the three states of thin filament suggested by McKillop and Geeves. The availability of myosin head atomic structure [127] and the atomic model of actin filament [128] made it possible to reconstruct the thin filament three-dimensional structure at a new level. Using electron microscopy, Lehman et al. reconstructed F-actin, TM, troponin, and S1 complexes in the presence and absence of calcium (cited by [129]). Thus, they managed to reveal three different positions of tropomyosin threads on the surface of the actin filament. It was shown that transition from state **B** to state **C** and then to state **M** is accompanied by displacement of the tropomyosin filament towards the "groove" of the actin filament. It was discussed above that the different positions of TM on the actin filament surface differ energetically due to the absence of specific contacts between actin and TM. This property of TM defines it as an ideal candidate for steric regulation – its position on the actin filament surface is easily changed and is defined by interactions with different proteins.

In conclusion of this part, it is interesting to consider the question concerning the influence of conformational mobility of the central part of TM on its regulatory functions. We managed to show that stabilization of the central part of TM by replacement of noncanonical residue Gly126 by Arg exhibits a noticeable effect on the regulatory properties of TM in fully reconstructed thin filaments (i.e. in filaments consisting of F-actin, TM, and troponin): this is revealed in a two-fold increase of S1 actin-activated ATPase activity at high calcium concentrations (at pCa = 5) [47]. The same effect was shown earlier in Lehrer's group in the case of the Asp137Leu mutation in central part of the α-TM molecule [46]. Thus, it can be concluded that this effect is caused just by stabilization of the central part of the TM molecule rather than by replacement of a certain amino acid residue. Based on the above-described theory of three states of thin filament [123], we suppose that two-fold increase of actin-activated myosin ATPase activity at high concentration of Ca^{2+} in the case of the Gly126Arg mutation is explained by the shift of equilibrium between states **C** (closed) and **M** (open) towards the latter [47]. It seems highly probable that it is just decreased conformational mobility that is responsible for the shift induced by TM mutations Gly126Arg or Asp137Leu. In fact, if actin-bound TM is more rigid (as this is supposed in the case of TM with mutations Gly126Arg or Asp137Leu) then this should enlarge the cooperative unit of the thin filament regulatory system. In this case, transition of one myosin head from the weak binding state to the state of strong binding, accompanied by translocation of TM on the actin surface, should "include" more adjacent sites on the actin filament for interaction with myosin and, correspondingly, a larger number of myosin heads will strongly bind actin. We believe that just such increased cooperativity of the interactions of myosin head with actin, caused by increased rigidity of the central part of TM, is responsible for noticeable increase of the ATPase activity under these conditions.

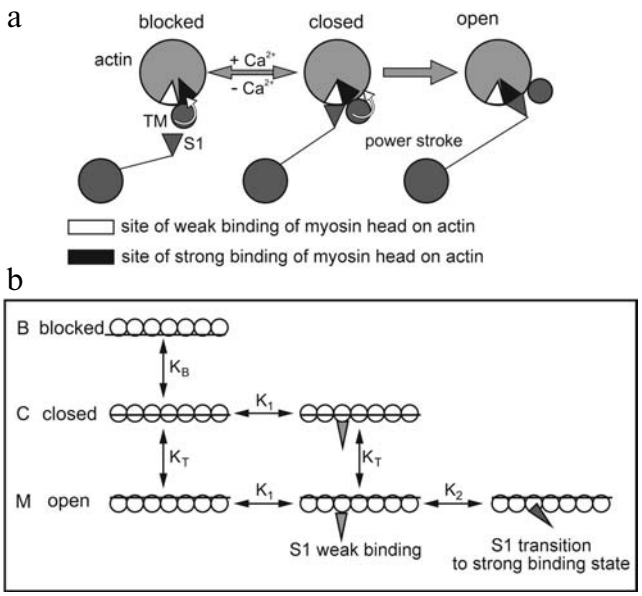

Fig. 5. Regulation of actin–myosin interaction with involvement of troponin–tropomyosin complex. a) Scheme of actin type regulation including three different states of thin filament ("blocked", "closed", and "open"). The end view of actin (above) and myosin (below) filaments is shown (according to [123]). b) Kinetic scheme of the "three state theory" (according to [123]; explanations are in the text).

MUTATIONS IN TROPOMYOSINS CAUSING MYOPATHY

Various effects of directed mutations in TM on its affinity to actin have been already discussed above. In this conclusions section we shall analyze functional effects of some chosen mutations in TM associated with development of hereditary muscle diseases. Hereditary factors are the basis of most cases of myopathy, i.e. of disturbances in muscle structure and functions. Among such diseases are nemaline myopathies [130], distal arthrogryposis [131], and familial cardiomyopathies in their two variants – hypertrophic (FHC, Familial Hypertrophic Cardio-

myopathy) and dilatational (DCM, Dilated Cardiomyopathy) [132, 133]. Nemaline myopathies are revealed as generalized muscle weakness and the presence of so-called nemaline bodies in skeletal muscle [130]. The frequency of nemaline myopathies is one case per 50,000 newborn [30]. Genetic analysis has shown that these myopathies are associated with mutations in β-TM (*TPM2*) and α-TM_{slow} (*TPM3* or γ-Tm) genes. The best-known mutations in α-TM_{slow} are Met9Arg and Arg167His. Definite progress in understanding the pathogenesis of nemaline myopathy was achieved with the appearance of transgenic mice carrying the Met9Arg mutation in the *TPM3* gene. It was shown that in slow muscle of such mice there is the upset ratio of heterodimeric and homodimeric TM forms, which can be a part of the mechanism of the development of the disease [30]. The main symptoms in the case of distal arthrogryposis are increased contractility (contracture) of muscle of extremities in the absence of any evident morphological or neurological changes [30, 131]. The overwhelming number of cases of arthrogryposis is associated with mutations in the β-TM (*TPM2*) gene, although there are also mutations in troponin genes. There is still practically no data about the mechanisms of development of these pathologies.

In the case of cardiomyopathy there are extensive morphological alterations of myocardial tissue. FHC is revealed as increased mass of cardiac muscle mainly due to left ventricle wall thickening (hypertrophy). As a result, the volume of the left ventricle cavity is smaller. Despite such pathological morphology, contractile function in the case of FHC rarely undergoes disturbance and sometimes it even exceeds norm by parameters; nevertheless, such cardiomyopathy in most cases results in cardiac insufficiency or sudden heart failure. The development of FHC can be associated with mutations in many contractile proteins (actin, TM, troponins, myosin heavy chain, myosin-binding C-protein) [132, 133]. In the case of TM, the most striking example of mutations associated with FHC is mutations Asp175Asn and Glu180Gly in the α-TM_{fast} (gene *TPM1*); numerous works deal with investigation of these mutations [134]. Some progress in understanding mechanisms of this type of cardiomyopathy was achieved in works by Watkins, Redwood, et al. They showed that the main functional effect combining FHC mutations is increased calcium sensitivity of TM (cited by [134]). The main characteristics of DCM are increased volumes of ventricle cavities, the thinning of their walls, arrhythmia, cardiac insufficiency, and in some cases death. It is characteristic that the DCM-associated mutations in TM (such as Glu40Lys and Glu54Lys in α-TM_{fast}), unlike FHC, cause decrease of calcium sensitivity in thin filament.

In most cases the reason for a particular myopathy is a mutation in regulatory proteins of thin filament. Because of this, investigation of structures and functions of proteins carrying such mutations can be useful for understanding mechanisms of development of myopathy and elaboration of approaches to their correction. Mutant proteins are a convenient model system for studying principles of structure and functioning of these molecules.

The main approaches for investigation of myopathic mutations in regulatory proteins (TM and troponins) include the characteristics of the functional properties of reconstructed thin filament. They include measuring calcium sensitivity of thin filament and actin-activated ATPase of myosin head as well as studying the rate of translocation of reconstructed thin filaments in the *in vitro motility* system. Such investigations often give ideas concerning general tendencies to changes in functioning of a thin filament containing a mutation in one of its components. Thus, in work by Marston et al. [135] in the case of all studied troponin T and C mutants a decrease of actomyosin ATPase activity and its sensitivity to calcium was observed. However, despite using all the numerous results of investigations, the exact reason for distortions of thin filament functioning often remains unclear. Just due to this, the most fruitful result is a combination of functional and structural approaches such as circular dichroism spectroscopy [136], the use of fluorescent labels [137], differential scanning calorimetry (DSC), and studying temperature dependences of dissociation of TM complexes with F-actin [55]. A good example of such combination is the work by Marston et al. [61] where the above-mentioned functional tests were successfully combined with DSC studies of the thermal unfolding of α-TM preparations carrying DCM mutations Glu40Lys and Glu54Lys and their complexes with F-actin.

There are also different examples of successful application of DSC in combination with other methods and approaches for investigation of TM species carrying myopathic mutations. Thus, it was shown that one (Asp175Asn) out of two FHC-associated mutations in α-TM has no significant effect on the thermal denaturation of F-actin-bound TM, whereas the other (Glu180Gly) noticeably decreases thermal stability of F-actin-bound TM and the temperature of its dissociation from the surface of actin filament [55]. These data for the first time made it possible to explain a serious difference between physiological effects of these mutations in the TM molecule. It is known that mutation Asp175Asn causes only light cardiac insufficiency, whereas mutation Glu180Gly results in severe cardiomyopathy, often in death. Our results suggest that when temperature in the heart increases by only a few degrees (like in the case of the common cold or intensive muscular activity), TM with the Glu180Gly mutation begins to dissociate from the actin filament surface and denature, and this results in severe consequences. No less interesting results have been recently obtained during investigation of β-TM with the Arg91Gly mutation associated with development of distal

arthrogryposis [62, 63]. It turned out that this mutation very strongly influences the structure and properties of β-TM: it causes significant structural alterations in its molecule, strongly lowering thermal stability of its N-terminal part [62, 63], significantly inhibits the ability of β-TM to form αβ-heterodimers with the TM α-isoform, and significantly decreases the affinity of β-TM to F-actin. All these facts show that β-TM with the Arg91Gly mutation is incapable of normal functioning in muscles, and this partially explains why this mutation in β-TM is associated with hereditary muscle disorder – distal arthrogryposis.

We finish this review with the conclusion that during time the concepts on TM structure and features of its interaction with actin have significantly changed. Although TM was previously considered as one of the most typical members the class of proteins with coiled-coil structure, this concept is now being actively revised. Unusual structural features specific only to TM are noted, and this is related to their most important functions in regulation of muscle contraction. A surprising property of the TM molecule is that, despite apparent structural homogeneity (fibrillar α-helical protein), properties of different parts of the molecule significantly differ from each other. According to present-day concepts, the TM molecule consists of two relatively stable and rather rigid domains (N- and C-terminal) joined by a flexible central region. High conformational mobility (flexibility) of the central part of TM and its effect on functional properties of the protein are now the subject of avid discussions. In particular, now there is continuing intensive discussion concerning the role of such flexibility in the interaction of TM with actin and in the translocation of TM on the actin filament surface, which are the basis of molecular mechanism of regulation of muscle contraction. Thus, the question of the structural–functional relationships in the TM molecule, which we have tried to describe in this review, now has no unambiguous answer and is intensively discussed in the present-day literature.

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