Conserved Noncanonical Residue Gly-126 Confers Instability to the Middle Part of the Tropomyosin Molecule*

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Tropomyosin (Tm) is a widespread actin-binding protein and a participant of regulation of muscle contraction. It is believed that many Tm functions are enabled by its flexibility whose nature has not been completely understood. We hypothesized that the well conserved non-canonical residue Gly-126 causes local destabilization of Tm. To test this, we substituted Gly-126 in skeletal muscle α-Tm either with an Ala residue, which should stabilize the Tm α-helix, or with an Arg residue, which is expected to stabilize both α-helix and coiled-coil structure of Tm. We have shown that both mutations dramatically reduce the rate of Tm proteolysis by trypsin at Asp-133. Differential scanning calorimetry was used for detailed investigation of thermal unfolding of the Tm mutants, both free in solution and bound to F-actin. It was shown that a significant part of wild type Tm unfolds in a non-cooperative manner at low temperature, and both mutations confer cooperativity to this part of the Tm molecule. The size of the flexible middle part of Tm is estimated to be 60–70 amino acid residues, about a quarter of the Tm molecule. Thus, our results show that flexibility is unevenly distributed in the Tm molecule and achieves the highest extent in its middle part. We conclude that the highly conserved Gly-126, acting in concert with the previously identified non-canonical Asp-137, destabilizes the middle part of Tm, resulting in a more flexible region that is important for Tm function.

The first and fourth (designated as a and d) residues are hydrophobic, whereas residues 5 and 7 (e and g) are charged. Residues a and d of two α-helices interact in a “knob-into-holes” manner (2), forming a continuous hydrophobic core, which glues the α-helices together. Charged residues in e and g positions form electrostatic interchain interactions and thus additionally stabilize the coiled-coil structure. Therefore, hydrophobic interactions (3, 4) and interchain electrostatics are the major determinants of Tm stability.

Tm coiled-coil structure is not perfect and contains some structural irregularities. Though being uninterrupted and non-overlapping, the heptad repeats of the Tm sequence (284 residues) include non-canonical residues in α or d positions, which are predicted to confer instability to Tm structure (e.g. Asp-137, Tyr-214, Gln-263, Tyr-267, as well as Ala, Ser, and other polar and charged residues in the interface). Generally, deviations from normal coiled-coil structure such as the presence of non-canonical residues, “skips” and “stutters” in heptad structure appear to be necessary for coiled-coil functioning, e.g. interactions with protein targets (5, 6).

It is believed that many Tm functions are enabled by its flexibility. Molecular flexibility is one of the basic concepts in protein science. Although frequently mentioned, it is not always clearly explained, so here we need to give a definition of this term. Flexibility is the conformational mobility of a molecule or its parts rising from a decreased rigidity of its ternary structure.

In terms of thermodynamics, it seems reasonable that the lack of rigid ternary structure should alter protein thermal unfolding, switching it from a classical “all-or-nothing” scheme to less well known non-cooperative mechanisms. This could be exploited for assessment of protein flexibility and disorder. To date, a solid body of data on Tm conformational flexibility exists in the literature (7–12). A study by Lehrer and co-workers (13) revealed that a particularly unstable region is located in the vicinity of the non-canonical residue Asp-137 because the peptide bond between Arg-133 and Leu-134 was shown to be the only site susceptible to cleavage by trypsin in the full-length Tm. This finding indicates that the region around Arg-133 is the least stable part of the molecule. Yet, the lack of proteolysis at potential cleavage sites adjacent to the other non-canonical residues (e.g. Gln-218) remains poorly understood.

In the present study, we focus on Gly-126, whose significance was omitted in earlier studies. Being a glycine in the g position, it should undermine the stability of Tm coiled coil, as glycine

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has the lowest helix-forming capacity, and it fails to form a stabilizing interchain salt bridge. We applied differential scanning calorimetry (DSC)² to investigate thermal unfolding of Tm mutants carrying stabilizing substitutions in position 126. We have shown that a considerable portion of Tm flexibility is associated with the middle part of the molecule. Our results indicate that increased flexibility of the middle part of Tm is attributed to the concerted action of two non-canonical residues, Asp-137 and Gly-126.

EXPERIMENTAL PROCEDURES

Protein Preparation—All of the Tms used in this work were recombinant proteins that have Ala-Ser N-terminal extension to imitate naturally occurring N-terminal acetylation of native Tm (14). Human TPM1 isoform 1 (α-striated Tm) G126A and G126R mutants were prepared in the bacterial expression plasmid pMW172 (15) by PCR-mediated site-directed mutagenesis using AccuPrime™ Pfx DNA Polymerase (Invitrogen). The oligonucleotides used for mutagenesis were as follows: GAGTGAGAGGCCATGAAG (for G126A) and GAGTGAGAGCGACATGAAG (for G126R; mutant codons are underlined). The PCR products were cloned and sequenced to verify the substitutions. The pMW172 constructs were used to transform the Escherichia coli strain BL21(DE3)pLysS, and large scale cultures were grown, and overexpression was induced according to standard methods (16). Bacterial cell lysates containing recombinant human Ala-Ser α-Tm were heated to 85 °C before clarification by centrifugation at 33,200 × g for 10 min. The resulting supernatant was fractionated by reducing mercaptoethanol with following preheating at 33,200 × g for 10 min. The resulting supernatant was fractionated by reducing mercaptoethanol with following preheating.

ATPase Measurements—Thin filament-induced activation of S1 ATPase was assayed in 10 mM Hepes, 50 mM NaCl, 5 mM MgCl₂, 5 mM β-mercaptoethanol, 0.002 mg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington) at 25 °C. At various times, aliquots were analyzed by SDS-PAGE sample buffer. Protein compositions of the aliquots were analyzed by SDS-gel electrophoresis.

DSC Measurements—DSC experiments on recombinant α-Tm WT, Tm G126A, and Tm G126R were performed as described earlier for α-Tm and its various mutants (21, 22). All Tm species were fully reduced before experiments by heating in the presence of β-mercaptoethanol with following preheating of the samples in the calorimeter cell. Protein-specific heat capacity (C_p) was calculated as described by Privalov and Potekhin (23).

Light Scattering—Thermally induced dissociation of Tm-F-actin complexes was detected by changes in light scattering at 90°, and the dissociation curves were analyzed as described earlier (21). Light-scattering measurements were performed under the same conditions and at the same heating rate as the DSC experiments.

CD Spectroscopy—Far-UV CD spectra of Tm species (2.0 mg/ml) were recorded on a Chirascan Circular Dichroism spectrometer (Applied Photophysics) in 0.02-cm cells. Thermal stability measurements were made by following the molar ellipticity of Tm at 222 nm as a function of temperature at a constant heating rate of 1 °C/min.

RESULTS

Design of Mutant Tropomyosins—Mutant design was based on the following considerations. Earlier, we showed that substitution of Arg-91 with glycine residue leads to dramatic destabilization of a considerable portion of the Tm molecule (24). This is not an unexpected result, taking into account the low helical propensity of Gly residues. However, what is surprising is the presence of a well conserved Gly residue at position 126, i.e. roughly in the middle of the Tm molecule. Assuming the destabilizing effect of Gly residues, we constructed two mutant proteins carrying stabilizing substitutions at position 126 to elucidate the impact of Gly-126 on the overall flexibility of the Tm molecule. One of the mutant proteins carries a G126A substitution, as Ala is known to have the highest helix-forming ability and, like Gly, is uncharged. The other mutant Tm contains a G126R substitution that is expected to stabilize both α-helix and coiled-coil structure due to formation of an interhelical salt bridge with Glu-131, between g of one heptad and e' of the following heptad on the other helix, termed i→i+5 (6). Therefore, the mutants G126A and G126R represent a gradual stabilization of some part of the Tm molecule, originally destabilized by the “native” Gly-126.

Tryptic Cleavage of Tm and Its Mutants, Tm G126A and Tm G126R—First of all, a Tm WT and its mutants, Tm G126A and Tm G126R, were subjected to limited tryptic digestion. Previous studies have shown that trypsin initially cleaves the Tm WT at Arg-133 into two fragments (25, 26), and the mutation D137L effectively prevents this cleavage (13). We observed very similar effects in the case of mutations G126A and G126R (Fig. 1). For Tm WT, the main band was fully lost after 1 h of incubation with trypsin, whereas the mutants Tm G126A and Tm G126R were not digested significantly (Fig. 1, A and B). These results are consistent with those of Sumida et al. (13) and suggest that mutations G126A and G126R stabilize, like mutation

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²The abbreviations used are: DSC, differential scanning calorimetry; Tm, tropomyosin(s); S1, myosin subfragment 1.
D137, a relatively unstable region in the middle of the Tm α-helix.

Functional Effects of Mutations G126A and G126R—Following previous studies of the Lehrer group on mutation D137L (13), we also investigated how mutations G126A and G126R affect functional properties of Tm. Actin binding properties of Tm WT and its mutants, Tm G126A and Tm G126R, were studied by co-sedimentation with F-actin. Neither mutation altered binding of Tm to actin, and both Tm mutants exhibited actin affinity similar to that of Tm WT (see supplemental Fig. 1). In this respect, the effects of mutations G126A and G126R were similar to those of mutation D137L, which had no influence on the actin-binding properties of Tm (13).

We also studied the effects of Tm mutants G126A and G126R on the Ca\(^{2+}\)-dependent ATPase of the fully regulated thin filament composed of F-actin, Tm, troponin, and myosin S1. At high Ca\(^{2+}\), the ATPase was activated almost 2-fold more for the Tm G126R mutant than the Tm WT (Fig. 2), and this effect was very similar to that observed earlier with Tm D137L mutant (13). In contrast, mutation G126A in Tm had no effect on the Ca\(^{2+}\)-dependent ATPase of the regulated thin filament (Fig. 2). Thus, there are some differences between Tm mutants in their effects on the Ca\(^{2+}\)-regulated thin filament.

Thermal Unfolding of Tm Carrying Mutations G126A and G126R—Measurement of the temperature dependence of CD at 222 nm showed that all three Tm species were fully folded at 5 °C and that their thermal stability was very similar, although some difference between Tm WT and its mutants was observed in a temperature region 30–65 °C. (The CD data are presented and discussed in the supplemental material.) To obtain more detail about the coiled-coil stability, we used DSC. The DSC profiles obtained for Tm WT, Tm G126A, and Tm G126R are presented in Fig. 3. The heat-induced unfolding of all Tm species was fully reversible. For both Tm mutants the major transition takes place at a higher temperature than for Tm WT (T\(_m\) = 44.3 °C), by 1.5 °C for Tm G126A and by 2.3 °C for Tm G126R. Both mutations significantly increased the magnitude of this transition. The main difference between the two Tm mutants was observed in the most thermostable transition, which remains almost unchanged in Tm G126A (T\(_m\) = 53 °C), but it shifts by >3 °C to a higher temperature for Tm G126R (Fig. 3).

To estimate, at least approximately, the enthalpy of Tm WT and its mutants, we extrapolated the heat capacity curves to the heat capacity expected for a globular protein having a stable and rigid structure; it was calculated according to Privalov and co-authors (27, 28) using the molecular mass of Tm.
unfolding of the fully folded state of the protein to its denatured state, were calculated to be 7600, 7200, and 6300 kJ/mol for Tm WT, Tm G126A, and Tm G126R, respectively. The decreased enthalpy of Tm G126R can be explained by its decreased heat capacity in a high temperature region, above 60 °C (Fig. 3). However, this effect cannot be attributed to incomplete unfolding of α-helix of Tm G126R, as its CD spectrum at 65 °C was identical to that of Tm WT (see supplemental Fig. 2A).

The heat capacity curves obtained for Tm WT and its mutants were subjected to deconvolution analysis as was described previously (21, 22), and the results show that each DSC profile can be decomposed into two separate thermal transitions (calorimetric domains) (Fig. 4). Previous studies on skeletal muscle α-Tm WT (21, 22, 29, 30) have shown that the least thermostable domain (domain 1 in Fig. 4A) corresponds to the thermal unfolding of C-terminal part of Tm with the thiols of Cys-190 reduced, and the most thermostable domain (domain 2 in Fig. 4A) corresponds to the N-terminal part of Tm. It is clear from Fig. 4 that both mutations, G126A and G126R, significantly increased (by ~80%) the calorimetric enthalpy (ΔH_cal) of domain 1, with a much less pronounced effect on the enthalpy of domain 2. Apart from the increased T_m value for N-terminal domain 2 in Tm G126R, the effects of mutations G126A and G126R were similar (see Table 1). Both of these mutations increased the total ΔH_cal value (by 47% for Tm G126A and by 27% for Tm G126R), mainly due to the significant increase in the enthalpy of C-terminal domain 1.

So far, our DSC data show that (i) the total energy of unfolding is roughly similar for all studied Tm species and that (ii) both mutations significantly increase the enthalpy of C-terminal domain 1. To reconcile these contradicting facts, we examined the initial slope of DSC profiles (see Fig. 3), which is much steeper in the case of the WT protein. The difference in slopes suggests that mutations G126A and G126R stabilize the middle part of the Tm molecule, which non-cooperatively unfolds below 30 °C in Tm WT, and thus make this part of the molecule unfold cooperatively at a higher temperature (46–47 °C); it either melts together with the C-terminal part, or its thermal transition coincides in position with that of the C-terminal domain 1, and the two transitions cannot be separated on the DSC profile.

To verify the assumption that non-cooperative unfolding of the middle part of Tm becomes cooperative in Tm G126A and Tm G126R, we studied the effects of F-actin on the thermal unfolding of these Tm mutants. Thermal Unfolding of Tm and Its Mutants in Presence of F-actin—It was shown earlier that Tm molecules bound to F-actin denature only after dissociation from actin filaments. In terms of DSC analysis, this is manifested in a new sharp peak on DSC profile, which reflects dissociation of F-actin-Tm complexes and denaturation of all those parts of Tm molecule that should have denatured at lower temperatures (21, 31, 32). Comparison of calorimetric profiles of actin-bound Tm with those of free Tm allows to evaluate the energetics of non-cooperative unfolding. This was demonstrated clearly in our previous DSC study on non-muscle Tms with non-cooperative unfolding of some their parts (31).

In the present work, we applied this approach to Tm G126A and Tm G126R with F-actin. Fig. 5 represents the results of deconvolution analysis of the DSC curve for Tm G126R (Fig. 5A) and temperature dependence of dissociation of the F-actin complex with the Tm mutant (Fig. 5B). Calorimetric domains 1 and 2 on Fig. 5A correspond to domains of actin-free

| Tm  | Domain 1 | Domain 2 |
|-----|----------|----------|
|     | T_m (°C) | ΔH (kJ/mol °C) | T_m (°C) | ΔH (kJ/mol) | Total ΔH_cal (kJ/mol) |
| WT  | 44.3     | 445      | 52.6     | 475         | 920               |
| G126A | 45.8    | 825      | 53.0     | 525         | 1350              |
| G126R | 46.6    | 795      | 56.4     | 375         | 1170              |

* The parameters were extracted from Fig. 4.

* The error of the given values of transition temperature (T_m) did not exceed ± 0.2 °C.

* The relative error of the given values of calorimetric enthalpy, ΔH_cal did not exceed ± 10%.

FIGURE 4. Temperature dependences of the excess heat capacity (C_p) and deconvolution analysis of the heat sorption curves of Tm WT (A), Tm G126A (B), and Tm G126R (C). The protein concentration was 30 μM. Other conditions were as follows: 20 mM Hepes, pH 7.3, 100 mM NaCl, and 1 mM MgCl_2. The heating rate was 1 K/min. The curves were analyzed according to the non-two-state model. Solid lines represent the experimental curves after subtraction of instrumental and chemical base lines, and dotted lines represent the individual thermal transitions (calorimetric domains 1 and 2) obtained from fitting the data to the non-two-state model.
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Tm G126R shown in Fig. 4C, whereas the new transition at 51.1 °C (peak AT) is attributed to the melting of actin-bound Tm. The temperatures of half-maximal dissociation of the Tm-F-actin complexes (T\text{diss}) for Tm WT, Tm G126A, and Tm G126R are listed in Table 2 and show a good correlation with T\text{m} values of peak AT (see Table 2). It is also seen from Table 2 that both Tm mutants dissociate from F-actin at higher temperatures (T\text{diss} \sim 50–51 °C) than Tm WT (T\text{diss} = 47 °C).

Knowing the enthalpies of separate calorimetric domains of Tm obtained in the presence or absence of F-actin (Table 2 versus Table 1), we were able to calculate the enthalpy of non-cooperative unfolding. Although some fraction of unbound Tm was present in the solution, it did not affect our calculations. First, we found differences between enthalpies of C-terminal domain 1 of Tm obtained in the presence or absence of F-actin (\(\Delta H_C(-\text{F-actin)} - \Delta H_C(+\text{F-actin)} = \Delta \Delta H_C\)) and the same for N-terminal domain 2 (\(\Delta \Delta H_N = \Delta H_N(-\text{F-actin)} - \Delta H_N(+\text{F-actin)}\) and compared the sum (\(\Delta \Delta H_C + \Delta \Delta H_N\)) with \(\Delta H_{\text{AT}}\). In theory, if the whole Tm molecule melts cooperatively, then (\(\Delta \Delta H_C + \Delta \Delta H_N\)) must be equal to \(\Delta H_{\text{AT}}\) because the decrease in enthalpies of domains is related to appearance of the new transition AT. However, if (\(\Delta \Delta H_C + \Delta \Delta H_N\) < \(\Delta H_{\text{AT}}\)), then it suggests that some part of actin-free Tm melts non-cooperatively, and therefore, its enthalpy cannot be registered by DSC, which only measures cooperative transitions. Binding to actin stabilizes Tm, so previously “unseen” enthalpy now contributes to the peak AT.

The estimated contribution of these parts of Tm toward the enthalpy of transition AT was 180 kJ/mol for Tm WT, whereas it was close to zero (within the error limits of \(\pm 10\%\)) for both Tm mutants. Thus, the DSC experiments on actin-bound Tm allowed us to estimate, albeit approximately, the enthalpy of non-cooperative unfolding occurring in Tm WT (but not in the Tm mutants) in the absence of F-actin. We calculated the enthalpy of this unfolding to be 320 ± 35 kJ/mol.

**DISCUSSION**

In the present work, we have addressed the role of the well-conserved amino acid residue Gly-126 in the structure of human skeletal muscle \(\alpha\)-Tm by means of trypsin digestion, DSC, and CD. Furthermore, the results obtained on skeletal \(\alpha\)-Tm were reproduced largely on another Tm isoform, \(\beta\)-Tm from smooth muscles (see supplemental Figs. 3–5), which proves the structural and functional significance of Gly-126. Our results show that the presence of a Gly residue at position 126 causes local Tm flexibility, which is manifested in high susceptibility to proteolysis.

The pattern of trypsin digestion of Tm and the initial cleavage site were described in earlier works by Pato et al. (25) and Ueno (26). Sumida et al. (13) attributed high susceptibility to trypsin cleavage at Arg-133 to the destabilizing effect of non-canonical Asp-137. However, it remained unclear why proteolysis did not occur in the vicinity of the other non-canonical

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**TABLE 2**

Calorimetric parameters of the thermal unfolding of Tm WT, Tm G126A, and Tm G126R in the presence of F-actin

The parameters were obtained from deconvolution analysis of the DSC curves obtained from the first heating of the complex of \(\alpha\)-Tm with phalloidin-stabilized F-actin. Concentration of Tm was 10 \(\mu\)M; concentration of phalloidin-stabilized F-actin was 45 \(\mu\)M. The error of the T\text{m} values did not exceed \(\pm 0.2\) °C. The relative error of the \(\Delta H_{\text{cal}}\) values did not exceed \(\pm 10\%\). Domains 1 and 2, as well as transition AT are described in the text. The values of T\text{diss} (the temperature of half-maximal dissociation of the TmF-actin complex) were calculated from light scattering experiments as it is shown in Fig. 5B. The error of the T\text{diss} values did not exceed \(\pm 0.2\) °C.

| Domain 1 | Transition AT | Domain 2 |
|----------|---------------|----------|
| Tm | \(T_m\) | \(\Delta H\) | \(T_{diss}\) | \(\Delta H\) | \(\Delta H_{\text{cal}}\) | \(T_{diss}\) |
| °C | kJ/mol | °C | kJ/mol | °C | kJ/mol | °C |
| WT | 43.6 | 200 | 46.7 | 520 | 52.5 | 380 | 1100 | 47.0 |
| G126A | 44.9 | 360 | 49.7 | 650 | 52.8 | 260 | 1270 | 49.9 |
| G126R | 45.9 | 300 | 51.1 | 520 | 53.7 | 320 | 1440 | 51.1 |
residues (Glu-218, Tyr-261, etc.) despite the presence of proper trypsin cleavage sites evenly scattered along the Tm molecule. We argue that the extent of Tm local flexibility sufficient for protein cleavage by the protease is achieved only by the concerted action of two non-canonical residues, Gly-126 and Asp-137, as the stabilizing substitution of either Asp-137 (13) or Gly-126 (Fig. 1) fully prevents trypsin cleavage at Arg-133. It is interesting to address the concerted action of Gly-126 and Asp-137 in terms of molecular architecture. Available atomic models of Tm segments (33) reveal a region of local chain separation in the vicinity of Asp-137. It seems possible that Gly-126 destabi-

lizes the individual chains causing their local unfolding and susceptibility to tryptic cleavage. Notably, cleavage does not occur at Gly-52 or Gly-188 because the coiled-coil chains do not splay out there. At the same time, Glu-218 and Gln-263 do cause local chain separation, but proteolysis still does not occur there due to the absence of chain-destabilizing residues (e.g. Gly).

In keeping with previous studies (21, 22, 29, 33), our data show that Tm WT melts in two roughly equal cooperative transitions, which are attributed to the unfolding of Tm N- and C-terminal parts (Fig. 4A and Table 1). A more careful examination of Tm WT thermal behavior in a low temperature range revealed that some part(s) of Tm unfold(s) with very low cooperativity as opposed to two highly cooperative main transitions (calorimetric domains). Our DSC results show that a stabilizing substitution at position 126 significantly reduces non-cooperative unfolding of Tm (Figs. 3 and 4), which therefore can be assigned to the middle part of the molecule. By measuring enthalpies of unfolding of actin-bound Tm and its mutants G126A and G126R, we were able to roughly estimate the enthalpy of non-cooperative unfolding of the middle part of the Tm. From this estimation, we could deduce the size of this part of the molecule, which should contain ~60–70 amino acid residues, i.e. about one-fourth of the Tm molecule.

Previous DSC studies on non-muscle Tms have revealed non-cooperative unfolding of significant parts of their molecules in comparison with muscle Tm (31). A principal result of the present study is that the most studied skelatal muscle α-Tm, which is often used as a “gold standard” in DSC experiments, also demonstrates non-cooperative unfolding of the rather extended region in the middle part of its molecule. Note that low cooperativity of unfolding should not be confused with low thermal stability, as this term is only applicable to cooperative thermal transitions and it reflects, in a narrow sense, the position of the calorimetric peak on the temperature scale.

Let us consider how low cooperativity of unfolding affects the structural properties of Tm molecule. It is obvious that non-cooperative unfolding is an attribute of a loosely packed, less rigid structure. Here, the family of intrinsically disordered proteins provides a good (although extreme) analogy because, in their case, the lack of defined tertiary structure is expressed in the low cooperativity of thermal unfolding (35, 36). The lack of rigid structure is ultimately manifested in high conformational mobility, i.e. flexibility as defined above. Therefore, we can conclude that the middle part of the Tm molecule exhibits a great extent of flexibility compared with its N- and C-terminal parts, and mutations G126A and G126R (and probably D137L) make this part of Tm more rigid (i.e. less flexible).

In terms of the functional effects of the stabilization of the Tm middle part, the present results exhibit a high similarity with those obtained by Lehrer and co-workers on D137L substitution (13). In common with Tm D137L (13), Tm mutants G126A and G126R did not differ from Tm WT in actin-binding properties (see supplemental Fig. 1). This means that the changes in flexibility of the Tm middle part have no effect on the actin affinity of Tm. Somewhat similar results were obtained by Sakuma et al. (34), who introduced a stabilizing substitution in the middle part of α-Tm. They showed that stability of the mutant protein as measured by DSC was increased (and therefore its flexibility was decreased), whereas actin binding basically was unaffected. Together with our results, these findings show that flexibility of the Tm middle part is not crucial for actin binding. As Tm flexibility does not appear to be the major determinant of actin-Tm interactions, other factors should be invoked to explain actin binding. One of them might well be the specific shape of Tm molecule corresponding to the helical contour of the actin filament, as is postulated in the so-called “Gestalt binding” model. However, this model in its original form (37) does not include Tm flexibility/structural instability, which seems to be an inherent Tm property (11, 13, 38). We assume, therefore, that flexibility, though inherent in Tm structure, is not a prerequisite for the actin binding function of Tm. In other words, flexibility and structural bias toward a particular shape are not mutually exclusive notions because having a particular shape does not mean it should be “frozen” and static. This concept is indeed very close to that suggested by Li et al. (12), although their model represents Tm as lacking structural instabilities and defects.

Our results, however, do not fully dismiss the functional importance of Tm flexibility. ATPase measurements revealed that the effect of G126R substitution on Ca\(^2+\) regulation of the thin filament (Fig. 2) is similar to that of the D137L mutation (13), so we can conclude that the effect is accounted for by the stabilization of the Tm middle part, and it is not a consequence of the particular amino acid substitution. However, the mechanism of this effect is quite unclear. Based on the three-state model of thin filament regulation (39, 40), we suggest that the 2-fold increase in ATPase rate at high [Ca\(^2+\)] in the case of Tm G126R can be explained by shifts of equilibria from the C (closed) to the M (open) state. We propose that decreased Tm flexibility could potentially account for this shift. In fact, if actin-bound Tm is more rigid, then it should augment the size of cooperative unit because now the attachment of one S1 head can activate more adjacent S1-binding sites (40). The observed difference between the mutants G126A and G126R (Fig. 2) might reflect a gradual decrease in flexibility of the Tm middle part. The mechanical basis of the Tm middle part flexibility can be chain slippage, as well as local unfolding of α-helix, the latter of which seem to be diminished by both mutations. However, only G126R should decrease chain slippage, working like a “safety pin” and joining the chains by a salt bridge. So, if the increase in ATPase rate is attributed to the stabilization of the Tm middle part, then the lack of such an effect in the case of Tm

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G126A is likely accounted for by insufficient stabilization of the coiled-coil structure.

To sum up, the principal result of the present study is the identification of a novel well conserved residue, Gly-126, that, in concert with Asp-137, accounts for the destabilization of the Tm middle part. We have also shown that this part of the molecule includes up to 60 residues and undergoes non-cooperative unfolding upon heating, which is a manifestation of the high flexibility of the Tm midregion. The N- and C-terminal domains of the Tm molecule also are flexible, or at least semi-flexible, due to the presence of so-called Ala clusters in the hydrophobic core (11), but the extent of this flexibility is incomparable with that of the middle part. We have also shown that binding to F-actin is not primarily attributed to flexibility of the Tm middle part but rather is accounted for by other factors (e.g. the shape of the Tm molecule). On the other hand, flexibility of the Tm middle part appears to be an important factor underlying the regulatory properties of Tm.

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