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Effects of two stabilizing substitutions, D137L and G126R, in the middle part of α-tropomyosin on the domain structure of its molecule

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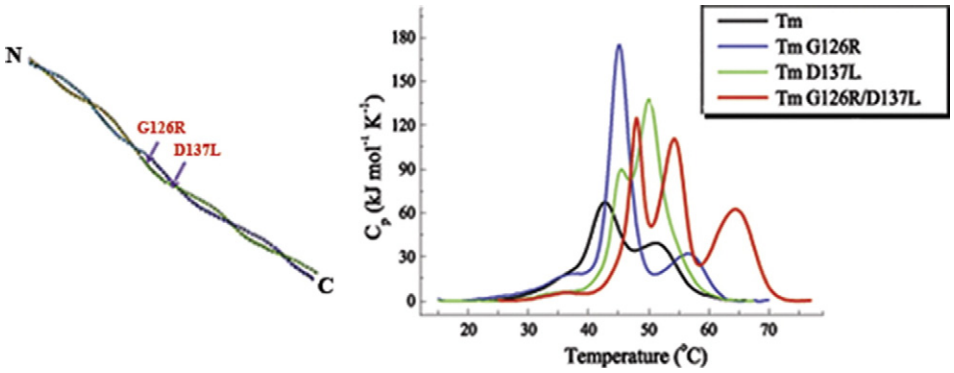
HIGHLIGHTS

- Non-canonical residues D137 and G126 destabilize the middle part of tropomyosin (Tm).
- These residues were replaced by canonical ones by mutations D137L and G126R.
- We applied DSC to study the effects of these mutations on the Tm thermal unfolding.
- These mutations (especially D137L/G126R) dramatically alter the Tm domain structure.
- Stabilization of Tm middle part can be transmitted to other parts of the molecule.

GRAPHICAL ABSTRACT



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ABSTRACT

We applied differential scanning calorimetry (DSC) to investigate the effects of substitutions D137L and G126R (i.e. replacement of conserved non-canonical residues Asp137 and Gly126 by canonical ones) in the middle part of tropomyosin (Tm), as well as the combined one, D137L/G126R, on the thermal unfolding of Tm. Special approaches (e.g. combination of DSC with measurements of temperature dependences of pyrene excimer fluorescence) were applied to assign separate thermal transitions (calorimetric domains) on the DSC profiles to the certain parts of Tm molecule. The results show that substitutions D137L and G126R (and, especially, the combined one, D137L/G126R) may stabilize not only the middle region of Tm, but also the other parts of its molecule including N- and C-terminal parts. These results suggest that the stabilization of the Tm middle part can be transmitted along the coiled-coil length displaying long-range effects.

1. Introduction

Tropomyosin (Tm) is an actin-binding α-helical protein that binds along the length of actin filament and is an integral component of thin filaments for Ca^{2+}-regulated contraction of striated muscle [1–4]. In

Abbreviations: DSC, differential scanning calorimetry; Tm, tropomyosin; β-ME, β-mercaptoethanol.

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terms of structure, Tm is a two-stranded coiled-coil protein. This structural motif is based on so-called heptad repeats — groups of 7 amino acid residues in which 1st and 4th (designated as *a* and *d*) residues are hydrophobic, while residues 5 and 7 (*e* and *g*) are charged. Residues *a* and *d* form 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 [4,5]. There are, however, some deviations from canonical coiled-coil structure, such as so-called "Ala clusters" [5] or charged residues in the hydrophobic core [4,6]. Such variations destabilizing the coiled-coil structure are predicted to confer conformational mobility (flexibility) to some parts of the Tm molecule and appear to be important for the Tm functioning.

In recent studies attention was attracted to two non-canonical residues naturally occurring in the middle part of skeletal α-Tm, Asp137 (a negatively charged Asp residue in the *d* position that is typically occupied by a hydrophobic residue) [6] and Gly126 in the *g* position instead of a typical charged residue [7]. Importantly, both Asp137 and Gly126 are highly-conserved, at least in all vertebrate Tm isoforms [8], emphasizing importance of these positions in the structure and function of Tm. To determine the role of these non-canonical residues, they were replaced with the canonical ones: Asp137 was replaced by Leu (D137L mutation), a highly preferred hydrophobic residue for the *d* position [6], and Gly126 — by a charged Arg (G126R mutation) [7]. It was shown that both these replacements have similar effects on structural and functional properties of Tm. They reduced the rate of characteristic Tm trypsinolysis between Arg133 and Leu134 in the middle part of Tm [6–8] and increased thermal stability of Tm analyzed by differential scanning calorimetry (DSC) [7,9,10] and circular dichroism (CD) [8]. Both substitutions stabilizing the middle part of Tm, D137L and G126R, at high calcium concentrations caused a significant increase in the actin-activated ATPase activity of myosin heads in the presence of regulated actin filaments (i.e. thin filaments reconstituted from F-actin, Tm, and troponin), although had almost no influence on the Tm affinity for actin [6–8]. Moreover, these stabilizing substitutions not only enhanced maximal sliding velocity of regulated actin filaments in the in vitro motility assay at high Ca^{2+} concentrations but also increased Ca^{2+}-sensitivity of the actin–myosin interaction underlying this sliding [11]. We proposed that the effects of these two substitutions on the Ca^{2+}-regulated actin–myosin interaction can be accounted for not only by decreased flexibility of actin-bound Tm but also by their influence on the interactions between the middle part of Tm and certain sites of the myosin head [8,11].

Importantly, in the previous study we for the first time analyzed the properties of Tm carrying both substitutions, D137L and G126R, within the same molecule and showed that the effects of their combined action on structural and functional properties of Tm are much more pronounced than the effects of each separate substitution: it was expressed in further significant stabilization of the α-Tm coiled-coil structure and in more marked effect on the actin–myosin interaction [8]. Moreover, it was proposed that mutations D137L and G126R (and especially double mutation D137L/G126R) can lead to significant changes in the domain structure of Tm molecule.

In the present work, in order to check this hypothesis and to get more insight into the coiled-coil stability in different parts of the molecule, we applied DSC as a basic method for the investigation of protein thermal unfolding [12–15]. This method has been extensively used to study the thermal unfolding of various Tm isoforms [16–21] and different Tm mutants [22–27]. An important advantage of this method is that it allows one to decompose a heat sorption curve into separate thermal transitions (calorimetric domains) corresponding to the melting of different parts of the Tm molecule, and thereby to determine how different mutations or modifications affect the structure of Tm. However, the presence of different calorimetric domains with peculiar behavior during thermal unfolding necessitated a more detailed analysis of the Tm domain structure as well as an explanation for its changes induced by D137L and G126R substitutions, including the identification of these domains (i.e. their assignment to certain regions of the molecule). For the latter purpose, special approaches (e.g. combination of DSC studies with measurements of temperature dependences of pyrene excimer fluorescence) were applied. Our results show that mutations D137L, G126R, and D137L/G126R may stabilize not only the middle part of Tm, where they are located, but also some other regions of the molecule including N- and C-terminal parts. These results suggest that the stabilization of the middle part of Tm by replacement of non-canonical residues Asp137 and Gly126 (and especially both of them) by canonical ones can be transmitted along the length of coiled-coil and affect other parts of the Tm molecule thus displaying long-range effects.

2. Materials and methods

2.1. Expression and purification of recombinant tropomyosins

All Tm species used in this work were recombinant proteins that have Ala–Ser N-terminal extension to imitate naturally occurring N-terminal acetylation of native Tm [28]. The cloning of C190A, D137L/C190A, G126R/C190A, and D137L/G126R/C190A mutant constructs of human *TPM1* isoform (α-striated Tm) into pMW172 vector [29] was described earlier [8]. The following oligonucleotides were used for mutagenesis:

5′-AAGGCAAAGCTGCCGAGCTTG-3′for C190A,

5′-GCCCAAAAACTTGAAGAAAAA-3′ for D137L,
and 5′-GAGTGAGAGACGCATGAAAG-3′ for G126R

(here and onwards mutant codons are underlined). The same primers were used to obtain Tm mutant constructs D137L, G126R, and D137L/G126R from wild type Tm containing endogenous Cys190. The Tm mutant constructs containing Cys36 instead of Cys190 (mutation S36C/C190A) were obtained from the above mentioned Cys-free Tm mutants C190A, D137L/C190A, G126R/C190A, and D137L/G126R/C190A with the oligonucleotide 5′-CAGCTGCTTGCACCTGTC-3′ used for S36C substitution. The PCR products were cloned into pMW172 vector and verified by DNA sequencing. The constructs were used to transform *Escherichia coli* BL21(DE3)pLysS cells. Large scale cultures were grown, and overexpression was induced according to standard methods [30]. Tm species were further obtained and purified substantially as described earlier [8] and Tm concentration was determined spectrophometrically at 280 nm using an $E^{1\%}$ of 2.7 cm^{-1}. According to SDS-PAGE [31], the purity of all preparations was no less than 95%.

2.2. Reduced and cross-linked tropomyosins

Tm species containing Cys residues may have been partially cross-linked upon storage due to the formation of disulfide bonds between the two Tm chains. To obtain these proteins in fully reduced state, they were heated at 60 °C for 45 min in the presence of 10 mM β-ME. To maintain the reduced Tm species 2 mM β-ME was added to the samples prior to DSC experiments [22]. The Tm species cross-linked by disulfide bonds were obtained by the use of 5,5′-dithiobis(2-nitrobenzoate) (DTNB) as was earlier described by S. Lehrer [32]. The content of cross-linked and reduced Tm dimers was estimated as described earlier [22], by SDS-PAGE [31] under non-reducing conditions, in the absence of β-ME.

2.3. Pyrene excimer fluorescence

Pyrenyliodoacetamide-labeled Tm was prepared by the method described by Ishii and Lehrer [33] with some minor modifications. In brief, reduced Tm was labeled specifically at its cysteine residues (either Cys190 or Cys36) with N-(1-pyrenyl)iodoacetamide (Invitrogen). The

reaction was carried out overnight at room temperature at 10 mol of reagent/mol of Tm, in 5 M GdmCl, 30 mM Hepes, pH 7.3, then the Tm samples were dialyzed against 30 mM Hepes, pH 7.3, containing 100 mM NaCl and 1 mM $MgCl_2$ to remove unreacted reagent. In all experiments the degree of Tm labeling with pyrene exceeded 50%. Fluorescence studies were performed on a Cary Eclipse fluorescence spectrophotometer (Varian) equipped with a Peltier-controlled cell holder and thermoprobes. Temperature dependences of pyrene fluorescence were recorded with a constant heating rate of 1 °C/min at Tm concentration of 1.0 mg/ml (15 μM). Fluorescence was excited at 340 nm (slit width 5 nm) and recorded at 390 nm (slit width 2.5 nm) for pyrene monomers and at 485 nm (slit width 2.5 nm) to monitor the changes in the pyrene excimer fluorescence. Upon heating, the pyrene monomer fluorescence decreased almost linearly because of the temperature-induced quenching, whereas the temperature dependences of decrease in pyrene excimer fluorescence reflected the dissociation of two Tm chains in the vicinity of a Cys residue (Cys190 or Cys36) to which the label was attached [33].

2.4. Differential scanning calorimetry (DSC)

DSC experiments were performed as described earlier [22,23] on a DASM-4 M differential scanning microcalorimeter (Institute for Biological Instrumentation RAS, Pushchino, Russia) at a 1 °C/min heating rate in 30 mM Hepes, pH 7.3, containing 100 mM NaCl and 1 mM $MgCl_2$. Protein concentration was 2.0 mg/ml. The reversibility of the heat sorption curves was assessed by reheating of the sample immediately after cooling from the previous scan. All Tms containing Cys190 or Cys36 were either fully reduced or almost completely cross-linked before DSC experiments. In the case of reduced Tm species the solution also contained 2 mM β-ME, and the DSC scans obtained from the first heating of these samples were omitted as this preheating was specially destined to reduce SH-groups of the protein and to prevent disulfide cross-linking between the chains in the Tm homodimers [22]. The calorimetric traces were corrected for the instrumental background by subtracting a scan with buffer in both cells. The temperature dependence of the excess heat capacity was further analyzed and plotted using Origin software (MicroCal Inc., Northampton, MA). The thermal stability of the proteins was described by the temperature of the maximum of thermal transition (T_m), and calorimetric enthalpy (ΔH_{cal}) was calculated as the area under the excess heat capacity function. Deconvolution analysis of the heat sorption curves, i.e. their decomposition into separate thermal transitions (calorimetric domains) was performed as described earlier [21–23].

3. Results and discussion

3.1. Thermal unfolding of recombinant α-Tm carrying mutations C190A, G126R/C190A, D137L/C190A, and D137L/G126R/C190A

The excess heat capacity curves obtained by DSC for recombinant α-Tm mutant constructs C190A, G126R/C190A, D137L/C190A, and D137L/G126R/C190A are presented in Fig. 1. The calorimetric traces of the C190A, G126R/C190A, and D137L/C190A Tm mutants were almost unchanged during a second heating of the samples. Therefore the thermal unfolding of these Tm species was fully reversible and can be considered to be at thermodynamic equilibrium, thus making possible deconvolution analysis of their DSC curves. In contrast, the thermal unfolding of Tm G126R/D137L/C190A was not fully reversible as a highly cooperative peak at 48 °C, which was clearly observed on the first heating of the sample, was not observed during the second heating when it melted with much lower cooperativity within a wide temperature range (Fig. 2). However, all following DSC curves of this Tm sample were identical, thus indicating that its thermal unfolding is reversible and deconvolution analysis of these curves is possible. Therefore in all following DSC studies of Tm carrying double mutation D137L/G126R in the middle part of its molecule we used for deconvolution analysis only the curves obtained from the second heating of the sample.

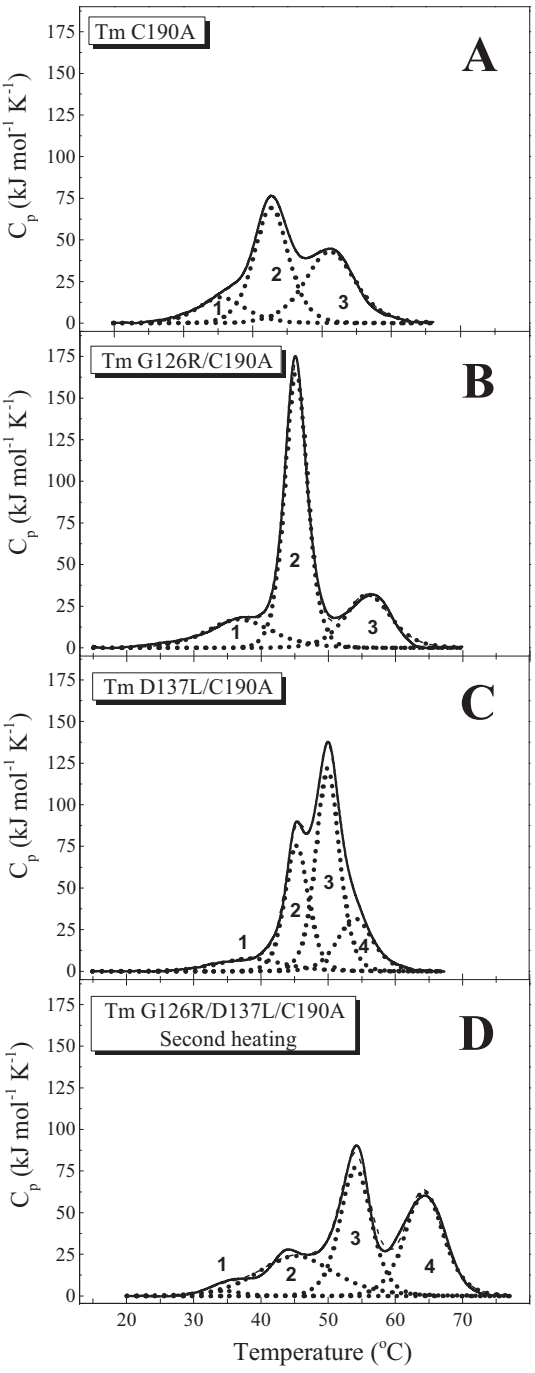

Fig. 1. Temperature dependences of the excess heat capacity (C_p) monitored by DSC and deconvolution analysis of the heat sorption curves of α-Tm mutant constructs C190A (A), G126R/C190A (B), D137L/C190A (C), and D137L/G126R/C190A (D). The protein concentration was 30 μM. Other conditions: 30 mM Hepes, 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 baselines, and dotted lines represent the individual thermal transitions (calorimetric domains) obtained from fitting the data to the non-two-state model.

In the previous work, we substituted the only Cys residue (Cys190) of α-Tm with an Ala residue to avoid potential complications due to possible disulfide cross-linking of two α-helices of the Tm dimer [8]. The results presented here show that the thermal unfolding of α-Tm C190A (Fig. 1A) is very similar to that obtained for α-Tm WT with the thiols of Cys190 reduced [22,23] (see also Fig. 3B). Both these Tm

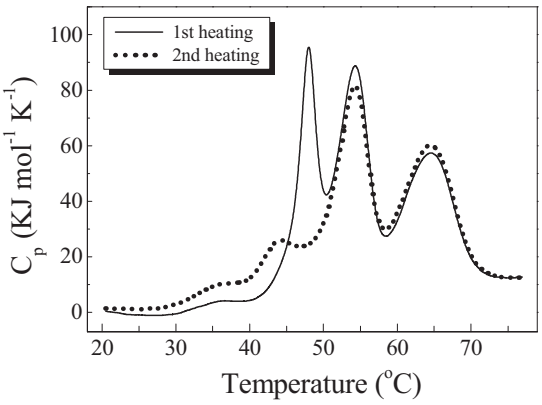

Fig. 2. Temperature dependences of the excess heat capacity of α-Tm mutant construct D137L/G126R/C190A obtained by DSC from the first (solid line) and second (dotted line) heating of the sample. Conditions were the same as in Fig. 1. (Note that in this case the DSC curves were not corrected by subtraction of chemical baselines).

species demonstrated three thermal transitions (calorimetric domains): low-cooperative transition with maximum at 35–36 °C, which presumably corresponded to some part of Tm with reduced thermal stability [23], and two high-cooperative transitions with maxima at 43–44 °C and ~51 °C (calorimetric domains 2 and 3 on Figs. 1A and 3B), which in previous studies were assigned to the thermal unfolding of C-terminal and N-terminal parts of α-Tm, respectively [16,22,23]. Thus, mutation C190A had no appreciable effect on the thermal unfolding of α-Tm, with the only exception that calorimetric domain 2 (C-terminal part of Tm) was somewhat less stable (by 1.5 °C) in Tm C190A than in reduced Tm WT (Tables 1, 2).

Fig. 1 shows also the results of deconvolution of DSC curves for α-Tm mutants C190A, G126R/C190A, D137L/C190A, and D137L/G126R/C190A into individual thermal transitions corresponding to separate calorimetric domains in the Tm molecule. The main calorimetric parameters for these domains (the maximum temperature of the transition, T_m, and calorimetric enthalpy, ΔH_{cal}, i.e. the area under the calorimetric peak) are summarized in Table 1.

In good agreement with previous DSC results [7], mutation G126R significantly increased (by ~25%) the calorimetric enthalpy of α-Tm C190A mainly due to a strong increase (by 65%) in the enthalpy of calorimetric domain 2, and increased the thermal stability of both cooperative transitions (by 2.5 °C for domain 2 and by 4.7 °C for domain 3 (Fig. 1B, Table 1)). Mutation D137L also increased (by 23%) the enthalpy of α-Tm C190A and caused significant changes in the domain structure of its molecule (Fig. 1C, Table 1). In this case, the heat sorption curve was decomposed into four calorimetric domains with maxima at 38.7, 45.4, 50.0, and 54.1 °C (Table 1). In this respect, our results differ from those earlier obtained by DSC for α-Tm D137L [10]. The authors revealed only two calorimetric domains in α-Tm D137L and suggested, rather reasonably, that mutation D137L increases thermal stability of both N- and C-terminal parts of Tm. However, they did not analyze at all the temperature region around 30 °C (i.e. the region of domain 1 thermal denaturation) and could not decompose the most thermostable transition into two domains. Our results presented in Fig. 1C suggest that the effect of mutation D137L is more complicated than it was proposed earlier [10].

The most surprising effect was observed with Tm carrying both substitutions, D137L and G126R within the same molecule (mutation D137L/G126R/C190A). This mutant protein demonstrated not only highly increased enthalpy (see Table 1) but also new high-temperature thermal transition at 64.3 °C (domain 4 in Fig. 1D), which has never been observed in previous DSC studies on Tm.

The above presented results show that mutations D137L and G126R in the middle part of Tm (and especially double mutation D137L/G126R) significantly alter the domain structure of its molecule and

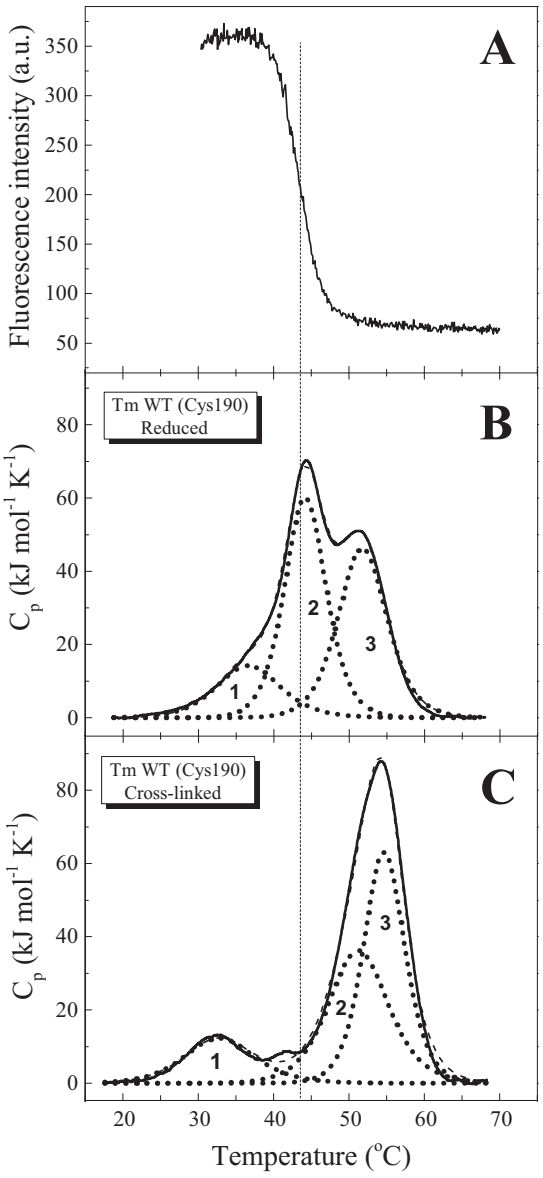

Fig. 3. (A) Temperature dependence of excimer fluorescence of pyrene label attached to Cys190 in α-Tm WT. (B, C) Temperature dependences of the excess heat capacity monitored by DSC and deconvolution analysis of the heat sorption curves of α-Tm WT in the reduced state (B) or in the cross-linked state (C). All measurements were carried out in 30 mM Hepes, pH 7.3, containing 100 mM KCl and 1 mM $MgCl_2$ with heating rate 1 K/min. Protein concentrations were 30 μM in DSC experiments and 15 μM in the fluorescence study.

strongly increase the calorimetric enthalpy of the thermal unfolding of Tm (Fig. 1, Table 1). The increase in the enthalpy can be explained by stabilizing effects of these mutations on the middle part of the Tm molecule, as it was proposed earlier for mutation G126R [7]. It was shown that the middle part of the α-Tm WT unfolds at low temperature (below 30 °C) in a non-cooperative manner, whereas mutation G126R stabilizes this part of the molecule and imparts cooperativity to its thermal unfolding [7]. As a result, the middle part of Tm stabilized by these mutations unfolds cooperatively at a higher temperature. It may either melt together with some other parts of the molecule, or its thermal transition may coincide in position with other calorimetric domains on the DSC profile, as it was earlier proposed for Tm with mutation G126R [7] which showed three such domains (see Fig. 1B). On the other hand, stabilized middle part of Tm may unfold as a separate cooperative thermal transition. It seems exactly the case with Tm mutants D137L and D137L/G126R which demonstrated four calorimetric domains on the DSC profile (Fig. 1C, D). Anyway, the increased calorimetric enthalpy

Table 1
Calorimetric parameters obtained from the DSC data for individual thermal transitions (calorimetric domains) of α-Tm mutant constructs C190A, G126R/C190A, D137L/C190A, and D137L/G126R/C190A[a].

Tm	T_m[b] (°C)	ΔH_{cal} (kJ mol^{-1})	ΔH_{cal} (% of total)	Total ΔH_{cal}[c] (kJ mol^{-1})
C190A				1000
Domain 1	35.5	140	14	
Domain 2	42.7	450	45	
Domain 3	51.2	410	41	
G126R/C190A				1245
Domain 1	36.8	220	17	
Domain 2	45.2	740	60	
Domain 3	55.9	285	23	
D137L/C190A				1230
Domain 1	38.7	95	8	
Domain 2	45.4	350	28	
Domain 3	50	565	46	
Domain 4	54.1	220	18	
G126R/D137L/C190A (second heating)				1300
Domain 1	34.4	15	1	
Domain 2	45.3	345	27	
Domain 3	54.1	470	36	
Domain 4	64.3	470	36	

[a] The parameters were extracted from Fig. 1.
[b] The error of the given values of transition temperature (T_m) did not exceed ±0.2 °C.
[c] The relative error of the given values of calorimetric enthalpy, ΔH_{cal}, did not exceed ±10%.

of Tm with mutations G126R, D137L, and D137L/G126R in the middle part of its molecule is most likely the result of stabilization of this part of the molecule.

It is clearly seen from the above presented results that the effects of stabilizing amino acid substitutions in the middle part of Tm on the thermal unfolding of its molecule are too complicated to be considered in the light of simple two-domain model including only two calorimetric domains corresponding to the N-terminal and C-terminal parts, which was generally accepted in previous DSC studies [7,10,16,22,23]. In order to examine the effects of these substitutions on the domain structure of Tm molecule more accurately, it was required to identify the thermal transitions on the DSC profiles, i.e. to assign them to the certain parts of the molecule.

Table 2
Calorimetric parameters obtained from the DSC data for individual thermal transitions (calorimetric domains) of α-Tm WT and its mutant constructs G126R, D137L, and D137L/G126R (in all the cases Cys190 was fully reduced), in comparison with the temperature of the half-maximal decay in excimer fluorescence of pyrene label attached to Cys190 in the C-terminal part of α-Tm (T_{ex}).

Tm	T_m[a] °C	T_{ex}[b] °C	ΔH_{cal} kJ mol^{-1} (% of total)	Total ΔH_{cal}[c] kJ mol^{-1}
WT				1000
Domain 1	36.7		160 (16%)	
Domain 2	44.2	43.6	440 (44%)	
Domain 3	51.8		400 (40%)	
G126R				1130
Domain 1	39.1		140 (12%)	
Domain 2	46.5	46.1	710 (63%)	
Domain 3	55.8		280 (25%)	
D137L				1070
Domain 1	38.2		250 (23%)	
Domain 2	46.3	46.9	230 (21%)	
Domain 3	50.0		490 (46%)	
Domain 4	54.0		100 (10%)	
G126R/D137L				970
Domain 1	32.2		80 (8%)	
Domain 2	47.0		250 (26%)	
Domain 3	54.1	53.5	260 (27%)	
Domain 4	62.8		380 (39%)	

[a] The error of the given values of transition temperature (T_m) did not exceed ±0.2 °C.
[b] The error of the given values of T_{ex} did not exceed ±1.0 °C.
[c] The relative error of the given values of calorimetric enthalpy, ΔH_{cal}, did not exceed ±10%.

3.2. Identification of calorimetric domains

We applied two special approaches to identify the calorimetric domains corresponding to C- and N-terminal parts of the Tm molecule on the DSC profiles of recombinant Tms with stabilizing substitutions G126R, D137L, and D137L/G126R in the middle part of the molecule. One of them was a combination of DSC studies with measurements of temperature dependence of decrease in pyrene excimer fluorescence reflecting separation of two Tm chains (i.e. local denaturation) in the vicinity of a Cys residue to which the label was attached [33]. This approach was successfully applied in previous studies to identify the calorimetric domain corresponding to the N-terminal part of smooth muscle β-Tm and its R91G mutant [24]. Another approach was a formation of the disulfide bond between Cys residues of the Tm dimer. This cross-linking was shown to increase the thermal stability of the Tm region where Cys residues are located [17,22]. Thus, the presence of a single Cys residue in different parts of the Tm molecule was required for both these approaches. For this purpose, a number of Tm mutant constructs containing the single Cys residue in different parts of Tm were produced, namely Tm with Cys36 in the N-terminal part (mutation S36C/C190A) and Tm WT containing Cys190 in the C-terminal part of the molecule. All these Tm mutants were used, together with above mentioned approaches, to investigate the effects of substitutions D137L, G126R, and D137L/G126R in the middle part of Tm on the domain structure of its molecule.

Fig. 3 illustrates how we applied both above mentioned approaches to identify calorimetric domains using α-Tm WT as an example. The decrease in excimer fluorescence of pyrene label attached to Cys190 occurs within the temperature range from 40 °C to 50 °C with a mid-point at 43.6 °C (Fig. 3A) and coincides in position on temperature scale with calorimetric domain 2 on the DSC profile of α-Tm WT with SH-groups of Cys190 in the reduced state (Fig. 3B). (Note that here and onwards temperature dependences of pyrene excimer fluorescence were compared with DSC data obtained for unlabeled Tms; the pyrenyliodoacetamide label had no appreciable influence on the thermal unfolding of Tm [33]). The thermal stability of this domain strongly increases in cross-linked Tm (Fig. 3C). All these indicate that calorimetric domain 2 can be assigned to thermal unfolding of the C-terminal part of α-Tm WT where Cys190 is located.

In following studies we applied these approaches to investigate how substitutions D137L, G126R, and D137L/G126R in the middle part of Tm affect the domain structure of α-Tm containing Cys190 in the C-terminal part of its molecule. In this case, however, we could not analyze the thermal unfolding of cross-linked samples by the following reason. All these Tm species demonstrated unusual and utterly unexpected effect of cross-linking between Cys190 residues on the thermal unfolding of Tm carrying mutations D137L, G126R, and D137L/G126R in the middle part of its molecule. This thermal effect was expressed in a dramatic increase of the protein heat capacity in a high-temperature region (above 60 °C and up to 95 °C), which was accompanied by a significant decrease in the enthalpy of the protein unfolding within the temperature range 40–60 °C. This unexplainable effect was irreversible as only thermal transitions with strongly decreased enthalpy were observed below 60 °C on the DSC profiles during reheating of the samples. All this fully prevented the analysis of the thermal unfolding and domain structure of Tm samples carrying mutations D137L, G126R, and D137L/G126R in the middle part of the molecule and cross-linked by disulfide bond between Cys190 residues of the Tm dimer. Therefore in this case we applied only combination of DSC studies on reduced Tm samples with measurements of temperature dependence of excimer fluorescence of pyrene label attached to Cys190. The results of these experiments are summarized in Table 2, in which the main parameters obtained by DSC for calorimetric domains (T_m, ΔH_{cal}) are compared

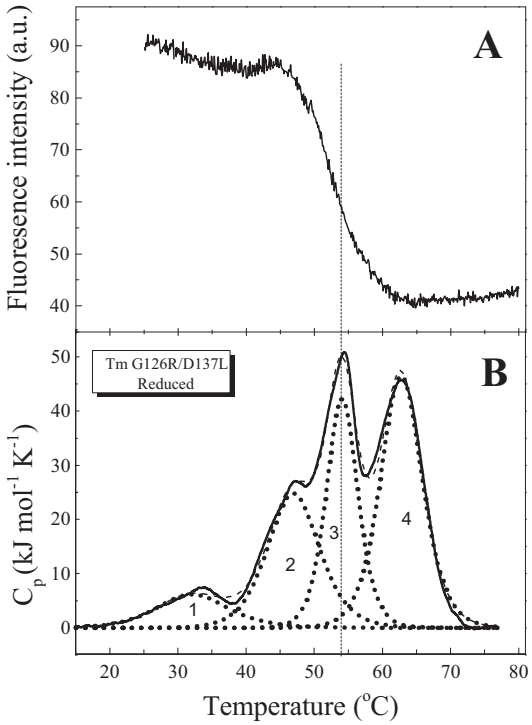

Fig. 4. (A) Temperature dependence of excimer fluorescence of pyrene label attached to Cys190 in α-Tm mutant construct D137L/G126R. (B) Temperature dependence of the excess heat capacity of α-Tm D137L/G126R in the reduced state and decomposition of the heat sorption curve into separate calorimetric domains. Conditions were the same as in Fig. 3.

with the temperature of the half-maximal decay (T_{ex}) in excimer fluorescence of pyrene label attached to Cys190 in the C-terminal part of α-Tm, and Fig. 4 shows the representative data obtained with α-Tm mutant construct D137L/G126R.

It should be noted that calorimetric parameters of individual thermal transitions obtained with Tm mutants containing Cys190 in the reduced state (Table 2) were very similar to those obtained for non-Cys Tms carrying mutation C190A (Table 1). Thus, the presence of SH-groups of Cys190 in their reduced state had no significant influence on the thermal unfolding and domain structure of the Tm molecule. It is clearly seen from Table 2 that the decrease in pyrene excimer fluorescence correlates well with the thermal unfolding of calorimetric domain 2 in Tm mutants G126R and D137L, and with the unfolding of domain 3 in the case of Tm D137L/G126R (Fig. 4). Thus, these calorimetric domains of Tm mutants can be assigned to thermal unfolding of the C-terminal part of the molecule where Cys190 is located.

The weakness of this approach, when it is applied without accompanying DSC analysis of cross-linked Tm species, is that in some cases it cannot unambiguously assign calorimetric domains to definite part of the molecule containing a pyrene-labeled Cys residue. For example, such a situation can be observed with Tm D137L/G126R (Fig. 4). In this case, the decrease in pyrene excimer fluorescence occurs within a rather wide temperature region, from 43 to 60 °C, that is the region of the thermal unfolding of two domains (not only domain 3, but also domain 2). This weakness is probably a typical property of the proteins for which the calorimetric domains cannot be clearly separated on the thermogram. On the other hand, it should be noted that Cys190 is located rather far from the C-terminus of the Tm molecule consisting of 284 residues, and therefore the excimer fluorescence of pyrene label attached to this residue can be sensitive not only to the thermal unfolding of the C-terminal part but also to the melting of a neighboring region, e.g. some region of the middle part of Tm stabilized by substitutions D137L, G126R, and D137L/G126R.

Nevertheless, comparing the T_{ex} values obtained for Tm WT and its mutant constructs D137L, G126R, and D137L/G126R (Table 2), we can conclude that these substitutions in the middle part of Tm significantly increase this parameter by shifting the curves for excimer fluorescence of pyrene label attached to Cys190 towards a higher temperature. Thus, substitutions D137L and G126R increased the T_{ex} value by 2.5–3.3 °C compared with Tm WT. However, the most pronounced effect was observed with the mutant Tm D137L/G126R for which the T_{ex} value (53.5 °C) is increased by almost 10 °C in comparison with Tm WT and by 6–7 °C in comparison with the Tm mutants D137L and G126R (Table 2). This probably means that both substitutions, D137L and G126R (and, especially, the combined one, D137L/G126R), increase the thermal stability of the C-terminal part of the Tm molecule (at least, its region where Cys190 is located).

The stabilizing effect of the combined substitution D137L/G126R was also the most pronounced in the case when the pyrene label was specifically attached to Cys36 in the N-terminal part of Tm (Fig. 5). In this case, the curve obtained for the temperature dependence of pyrene excimer fluorescence was drastically shifted to a higher temperature, and the T_{ex} value (67.1 °C) was increased by more than 12 °C in comparison with that for Tm WT (54.8 °C) (Table 3). On the other hand, the effect of mutation G126R was much less pronounced (the T_{ex} value was increased by only 2.2 °C in comparison with Tm WT), while no effects were observed with Tm D137L mutant (Fig. 5, Table 3). As mentioned above, the decrease in pyrene excimer fluorescence reflects the separation of two Tm chains, i.e. their denaturation, in the vicinity of a Cys residue to which the label is attached (Cys36 in this case). Thus, the above presented results (Fig. 5) indicate that combined substitution D137L/G126R dramatically increases the thermal stability of the N-terminal part of Tm containing Cys36, single mutation G126R only slightly stabilizes this region of the molecule, whereas mutation D137L has almost no effect on its stability.

By comparing the results of the pyrene fluorescence experiments with the DSC data obtained on Tm samples with the SH-groups of Cys36 in reduced state (upper plots on Fig. 6A–D, see also Table 3), we can suggest that the most thermostable calorimetric domain (domain 3 in Tm mutants S36C/C190A and S36C/G126R/C190A, and domain 4 in Tm S36C/D137L/C190A and Tm S36C/G126R/D137L/C190A) most likely corresponds to the thermal unfolding of the N-terminal part of Tm where Cys36 is located.

An assignment of the most thermostable calorimetric domain on the DSC profiles of Tm species with mutation S36C/C190A to the N-terminal part of Tm containing Cys36 is corroborated by the results of cross-linking experiments (lower plots on Fig. 6A–D, see also Table 3). Unlike the above described situation with Tm mutants containing Cys190,

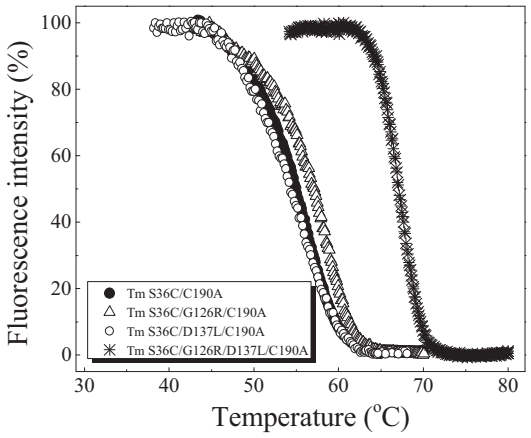

Fig. 5. Normalized temperature dependences of excimer fluorescence of pyrene label attached to Cys36 in α-Tm mutant constructs S36C/C190A, S36C/G126R/C190A, S36C/D137L/C190A, and S36C/D137L/G126R/C190A. Conditions were the same as in Fig. 3A.

Table 3
Calorimetric parameters (T_m and ΔH_{cal}) obtained from the DSC data for individual thermal transitions (calorimetric domains) of α-Tm mutant constructs S36C/C190A, G126R/S36C/C190A, D137L/S36C/C190A, and D137L/G126R/S36C/C190A, in either reduced or cross-linked state of Cys36 in the N-terminal part of Tm. The parameters T_m and ΔH_{cal} were extracted from Fig. 6, and parameters T_{ex} (the temperature of the half-maximal decay in excimer fluorescence of pyrene label attached to Cys36) were extracted from Fig. 5.

Tm mutant construct and state of Cys36, reduced or cross-linked	T_m[a] °C	T_{ex}[b] °C	ΔH_{cal} kJ mol^{-1} (% of total)	Total ΔH_{cal}[c] kJ mol^{-1}
S36C/C190A, reduced				1010
Domain 1	36.3		130 (13%)	
Domain 2	43.4		480 (47%)	
Domain 3	56.1	54.8	400 (40%)	
S36C/C190A, cross-linked				930
Domain 1	34.8		110 (12%)	
Domain 2	42.7		460 (49%)	
Domain 3	68.9		360 (39%)	
G126R/S36C/C190A, reduced				1060
Domain 1	37.3		70 (7%)	
Domain 2	46.7		590 (56%)	
Domain 3	57.9	57.0	400 (37%)	
G126R/S36C/C190A, cross-linked				950
Domain 1	36.5		100 (10%)	
Domain 2	44.3		580 (61%)	
Domain 3	67.8		270 (29%)	
D137L/S36C/C190A, reduced				1050
Domain 1	37.7		160 (15%)	
Domain 2	47.0		300 (29%)	
Domain 3	51.1		200 (19%)	
Domain 4	55.9	54.3	390 (37%)	
D137L/S36C/C190A, cross-linked				920
Domain 1	36.0		170 (18%)	
Domain 2	46.4		480 (52%)	
Domain 3	67.2		270 (30%)	
G126R/D137L/S36C/C190A, reduced				1250
Domain 1	36.9		160 (13%)	
Domain 2	49.5		250 (20%)	
Domain 3	53.9		350 (28%)	
Domain 4	65.4	67.1	490 (39%)	
G126R/D137L/S36C/C190A, cross-linked				1030
Domain 1	34.4		50 (5%)	
Domain 2	45.9		180 (17%)	
Domain 3	54.0		420 (41%)	
Domain 4	70.1		380 (37%)	

[a] The error of the given values of transition temperature (T_m) did not exceed ±0.2 °C.
[b] The error of the given values of T_{ex} did not exceed ±1.0 °C.
[c] The relative error of the given values of calorimetric enthalpy, ΔH_{cal}, did not exceed ±10%.

which demonstrated extraordinary effects of cross-linking, this approach was applicable to Tm species containing Cys36 in the fully cross-linked state. The results of the experiments clearly showed that in all the cases the formation of a disulfide bond between Cys36 residues in the Tm dimer strongly increases the value of transition temperature (T_m) for the most thermostable calorimetric domain (Table 3) by shifting its thermal transition towards higher temperature (Fig. 6). Interestingly, the least pronounced effect of the cross-linking was observed with Tm carrying combined substitution D137L/G126R, for which the T_m value of domain 4 increased only by 4.7 °C in comparison with the reduced sample (Fig. 6D), whereas for all other Tm mutants the thermal stability of domain 3 (or domain 4 in the case of mutation D137L) increased by 10–12 °C (Fig. 6A–C, Table 3). This effect can be explained as follows. The N-terminal part of Tm is already so strongly stabilized by the substitution D137L/G126R in the middle region of the molecule that its further stabilization by the cross-linking appears to be rather difficult in comparison with the other Tm mutants.

Thus, the most thermostable calorimetric domain (domain 3 in Tm with mutation G126R and domain 4 in Tm with mutations D137L or G126R/D137L) can be almost unambiguously assigned to the thermal unfolding of the N-terminal part of Tm. These results are somewhat contrary to those obtained earlier by DSC for Tm with mutation D137L [10], where calorimetric domain 2 at 52 °C was assigned by the authors to the N-terminal part of Tm. In our study their domain 2 was decomposed into two domains (domains 3 and 4), and only one of them (the most thermostable domain 4 on Figs. 1C and 6C) can indeed be assigned to the thermal unfolding of the N-terminal part of Tm.

The situation is more complicated in the case of the C-terminal part of Tm, which probably unfolds as domain 2 in Tm D137L or domain 3 in Tm D137L/G126R on the DSC profiles presented in Figs. 1C, D and 6C, D. However, in is not excluded that some regions of the C-terminal part of Tm may unfold together with other regions of the molecule (e.g., the middle region stabilized by these mutations). Therefore it is important to elucidate how the middle part of Tm unfolds when it is stabilized by mutations G126R, D137L, or D137L/G126R.

As proposed earlier [7] and discussed above, stabilized middle part of Tm may unfold as a separate cooperative thermal transition. In favor of this assumption is an appearance of new calorimetric domains on the DSC profiles of Tm mutants D137L and D137L/G126R, namely domain 3 for Tm D137L (Fig. 1C and upper plot on Fig. 6C) and domain 2 for Tm D137L/G126R (Figs. 1D, 4, and upper plot in Fig. 6D), which could not be assigned with enough certainty to the C-terminal part of Tm as was suggested above. It seems likely that these domains can be assigned to the thermal unfolding of the middle part of Tm stabilized by substitutions D137L and D137L/G126R. In line with this assumption are the DSC data showing that the formation of a disulfide bond between Cys36 residues in the Tm dimer decreases the thermal stability of both these domains. As a result, domain 3 in Tm D137L fully disappears on the thermogram and most likely coincides in position with neighboring less stable domain 2 whose enthalpy strongly increases in the cross-linked state (Fig. 6C), whereas the transition of domain 2 in Tm D137L/G126R shifts by 3.6 °C towards a lower temperature (Fig. 6D). It was shown in previous studies that the formation of a disulfide cross-link at C190 increased at elevated temperature (35–40 °C) the rate of tryptic cleavage at Arg133 in the middle region of Tm [34,35]. This probably means that the cross-linking (at Cys36 in our case) may decrease the thermal stability of the middle part of Tm even if it is stabilized by substitutions D137L or D137L/G126R. If so, these two domains, domain 3 in Tm D137L and domain 2 in Tm D137L/G126R can be assigned to the melting of the middle part of Tm. As to Tm with mutation G126R, its middle region most likely unfolds together with the C-terminal part of Tm as a single calorimetric domain with strongly increased enthalpy (domain 2 on Fig. 1B, Table 1), whose transition shifts, in the cross-linked state, by more than 2 °C towards a lower temperature (Fig. 6B).

The assignment of the calorimetric domain 2 on the DSC profile of Tm D137L/G126R to the thermal unfolding of the middle part of Tm is also corroborated by unusual features of this domain, i.e. its incomplete reversibility during reheating (Fig. 2). This strange effect can be explained as follows. The middle part of Tm is too strongly stabilized by the combined substitution D137L/G126R, and this is expressed in a highly cooperative peak at 48 °C clearly observed on the DSC profile (see the curve obtained from the first heating of the sample in Fig. 2). However, it seems probable that this stabilization was so strong that the middle region of Tm could not fully refold after denaturation, and therefore it only demonstrated a low-cooperative transition on the curve obtained from the second heating of the sample (Fig. 2). At the same time, we cannot exclude that the thermal unfolding of the Tm middle part is still reversible, but the refolding is kinetically limited and may require a rather long time.

4. Conclusions

In this paper we have investigated the effects of two stabilizing substitutions in the middle part of Tm, D137L and G126R, as well as combined substitution D137L/G126R, on the domain structure of Tm molecule. For this purpose, we have applied special approaches to identify the calorimetric domains on the DSC profiles of recombinant Tm

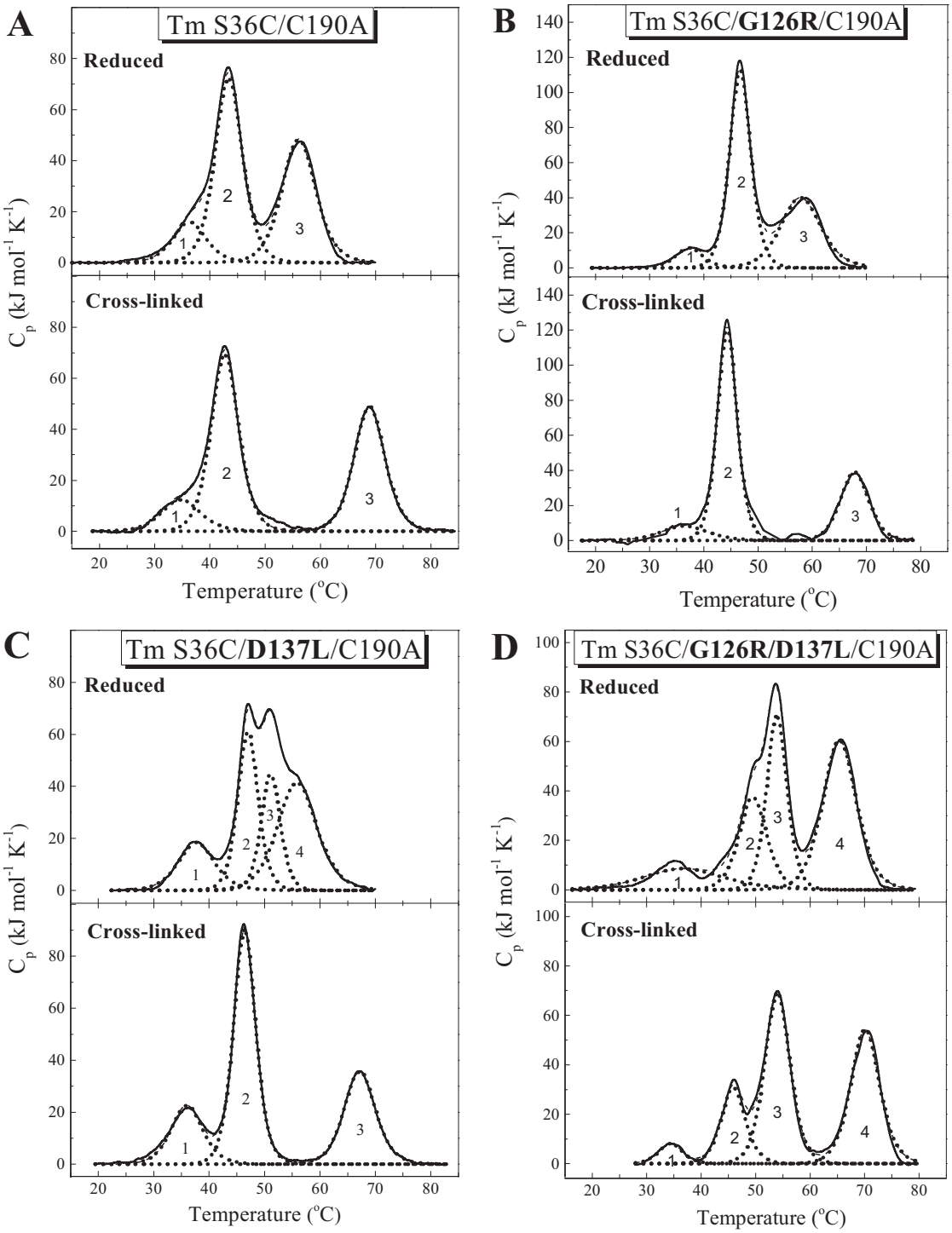

Fig. 6. Temperature dependences of the excess heat capacity of α-Tm mutant constructs S36C/C190A (A), S36C/G126R/C190A (B), S36C/D137L/C190A (C), and S36C/D137L/G126R/C190A (D) in the reduced state (upper plots) and in the cross-linked state (lower plots) of Cys36 in the N-terminal part of the α-Tm molecule. Solid lines represent the experimental curves after subtraction of instrumental and chemical baselines, and dotted lines represent the individual thermal transitions (calorimetric domains) obtained from fitting the data to the non-two-state model. Protein concentrations were 30 μM. Other conditions were the same as in Fig. 1.

species carrying these substitutions, i.e. to assign them to the certain parts of the Tm molecule. The main results show that substitutions D137L and G126R (and, especially, the combined one, D137L/G126R) may stabilize not only the middle region of Tm, where they are located, but also other parts of the molecule including N- and C-terminal domains. For example, unusual high-temperature thermal transition at 63.5 °C, which was observed by DSC for Tm G126R/D137L, was assigned to the N-terminal part of the Tm molecule strongly stabilized by this mutation in the middle part of Tm. Overall, our results suggest that stabilization of the middle part of Tm by replacement of non-canonical residues Asp137 and Gly126 (and especially both of them) by canonical ones can be transmitted along the length of coiled-coil thus displaying long-range effects. The stabilization which makes the Tm molecule more rigid, i.e. less flexible, may at least partly explain the effects of investigated substitutions on the functional properties of Tm, namely a significant increase both in the maximal sliding velocity of regulated

actin filaments in the in vitro motility assay and in the Ca^{2+}-sensitivity of the actin-myosin interaction underlying this sliding [8,11].

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