Classification of tropomyosin components into an α-like or a β-like family by partial peptide mapping

Elissavet Kardami and Marc-Yves Fiszman*

Institut Pasteur, Biologie Moléculaire, 28 Rue du Dr Roux, 75724 Paris Cedex 15, France

Tropomyosins can be classified as belonging to an α-like or a β-like family depending on the absence or presence, respectively, in their protease-V8 digestion pattern of two peptides with an apparent molecular mass of 21 kDa. Chicken cardiac tropomyosin and the 43 kDa component from gizzard tropomyosin are accordingly classified as α-like tropomyosins, while the 33 kDa gizzard tropomyosin component is a β-like tropomyosin. The 21 kDa peptides have an overall charge which is more positive than that of the intact tropomyosin or any other tropomyosin peptide and probably contain the -NH$_2$ half of the molecule.

Tropomyosin classification   *Partial peptide mapping*   *β-Tropomyosin 'difference' peptide*

1. INTRODUCTION

Tropomyosin is an essential component of the muscle contractile apparatus, interacting with actin and the troponin complex in the thin filament (review [1]).

Muscle tropomyosin exists as a homo- or hetero-dimeric coiled coil with a molecular mass of 68 kDa [2]. Two major monomeric forms have been described, employing as a criterion for identification their relative mobility in the SDS–PAGE system. The faster migrating component within a single muscle type has been termed α-tropomyosin while the slower migrating component has been termed β-tropomyosin [3]. In the case of rabbit skeletal muscle, both α- and β-tropomyosins consist of 284 amino acids, differing from each other in 39 positions [4,5]. No functional differences have as yet been demonstrated between α- and β-tropomyosins.

* To whom correspondence should be addressed

We have undertaken the comparison of the different tropomyosin components, in the avian and the mammalian system. A question we have addressed in particular was whether the relative electrophoretic mobility is a criterion sufficient for the description of an as yet uncharacterized tropomyosin component as an α-like or a β-like tropomyosin.

We have developed a simple procedure for the isolation of small quantities of tropomyosin components, at a high degree of purity, followed by partial proteolytic cleavage and analysis in a slab or a 2-D gel system.

We show that the *Staphylococcus aureus* proteinase (protease-V8) digestion patterns from all tropomyosins examined, can be confidently classified into two groups corresponding to an α-like or a β-like family of tropomyosins, depending on the absence or presence, respectively, of a characteristic doublet band, with an apparent molecular mass of 21 kDa.

We also show that in the case of smooth muscle tropomyosin from chicken, the faster migrating component which had been conventionally called α-tropomyosin, gives a characteristic β-like digestion pattern, while the slower migrating component gives a characteristic α-like digestion pattern.

The tropomyosin components from chicken heart muscle and the 30 kDa tropomyosin from pig platelets also produce an α-like digestion pattern.

Finally, we show that the 21 kDa doublet, produced by all the β-like tropomyosins in the avian and the mammalian system, represents two peptides with a higher accumulation of positive charges than either the intact or the rest of β-tropomyosin or the α-tropomyosin molecule. Calculations based on the amino acid sequence of β-tropomyosin from rabbit [5], indicate that these peptides may contain the -NH₂ half of the molecule.

2. MATERIALS AND METHODS

2.1. Tropomyosin preparation

Tropomyosin was prepared from adult chicken muscles as in [3], with minor modifications. Isoelectric precipitation was affected at pH 4.5 for all preparations. In the preparations from cardiac and gizzard muscles, the bulk of tropomyosin precipitated between 30–40% and 50–70% ammonium sulphate saturation, respectively. Pig platelet tropomyosin was a generous gift from Dr E. der Terossian. Lyophilized rabbit tropomyosin was purchased from Sigma.

2.2. Tropomyosin fractionation

Electrophoretic separation of tropomyosin components combines speed and purity of preparation. Several contaminating proteins may nevertheless comigrate with tropomyosin in any type of slab gel system employed: troponin-T and actin comigrate with β-tropomyosin from skeletal muscle and the 43 kDa component from gizzard, respectively, in the standard SDS–PAGE system, while α-tropomyosin and the 33 kDa gizzard tropomyosin comigrate with actin when urea is included in the standard system. The various tropomyosin components were therefore isolated after two cycles of small-scale preparative electrophoresis, as follows:

(i)   About 250–500 μg of the crude tropomyosin preparation (depending on the number of the different tropomyosin components in each preparation) were fractionated electrophoretically, in the presence of 0.1% SDS and 3.5 M urea, in a 12.5% acrylamide, 0.1% bis-acrylamide, 0.8 mm thick, slab gel system

[6]. The tropomyosin-containing bands were excised from the gel after staining for 10 min with a Coomassie blue (Bio Rad) solution, followed by destaining for 15 min in 40% methanol, 10% acetic acid.

(ii)   Each tropomyosin-containing gel strip was equilibrated for 15 min in the stacking gel buffer [6], containing 10% glycerol, and was subsequently placed on top of the stacking gel, in a 1 mm thick gel system identical to the one described in step (i) but omitting the urea in the gel solution. The tropomyosin bands in the second gel were visualized using the 'KCl-staining' technique [7].

(iii)   Tropomyosin elution: each tropomyosin-containing gel strip was cut to 1–2 mm long pieces and was incubated overnight with 2 ml of Cleveland-digest buffer (Tris, 0.125 M, pH 6.8, EDTA, 1 mM, SDS, 0.1%) [8], at room temperature, under mild agitation. This was repeated twice. The tropomyosin content in 50 μl of the total eluate was 1.5–2 μg of tropomyosin (up to 80% elution yield).

2.3. Partial-peptide mapping

Fifty μl of the tropomyosin-containing eluates were incubated with either 0.1–0.3 μg of chymotrypsin (Worthington) or 0.001–0.05 μg of Staphylococcus aureus protease-V8 (Miles), for 2 h at 37°C. The reaction was stopped with the addition of 12 μl of a 10% SDS, 50% β-mercaptoethanol solution per digest, followed by 5 min in a boiling water bath.

The digets were routinely analyzed in a 12.5% acrylamide, 0.3% bis-acrylamide slab gel system [6]. M_r-markers, purchased from Bio-Rad, were always included in each run. When 2-D analysis [9] of the digests was required, proteolytic digestion was arrested by the addition of urea, NP-40, ampholines and β-mercaptoethanol [9], and the digests were analyzed using an ampholine range of pH 4–6 (LKB) for the first dimension, and a slab gel system like the one described in the beginning of this paragraph for the second dimension.

The digestion patterns were visualized using a silver-staining procedure [10], omitting the glutaraldehyde step.

3. RESULTS AND DISCUSSION

The tropomyosin components from the various chicken muscles can be distinguished according to their electrophoretic mobility in the standard SDS−PAGE system [6], in the absence and presence of urea [11]. The faster migrating component within any one system has been conventionally termed α-tropomyosin and the slower migrating one, β-tropomyosin [3]. In the case of gizzard tropomyosin therefore, the components migrating with an apparent molecular mass of 43 and 33 kDa (the 43K and the 33K components) have been considered as β-tropomyosin and α-tropomyosin respectively, from smooth muscle [12].

Although polyacrylamide gel analysis can generally discriminate between the various tropomyosin components within any one system, partial-peptide mapping is indispensable for detecting possible structural differences between components that may comigrate in gels, as we have found to be the case for the β-tropomyosins from fast and slow chicken skeletal muscles [13].

When chymotrypsin is used for the partial cleavage of chicken tropomyosins, it is evident that components which differ in their electrophoretic mobilities exhibit a characteristic individual digestion pattern [13]. This can again be observed in fig.1 where the digestion pattern of cardiac and smooth muscle tropomyosins is shown, in comparison to that of their skeletal muscle counterparts. Chymotrypsin produces relatively large-sized peptides and no conclusions can be drawn concerning similarities in the patterns of the various tropomyosins.

When protease-V8 is employed (fig.2), each tropomyosin component examined again produces its own characteristic digestion pattern. Certain similarities in the patterns can nevertheless be immediately observed. While the bulk of the peptides accumulate at the lower-M_r region, there appears a closely spaced doublet, with an apparent molecular mass of 21 kDa (the 21K doublet) which is always present in some of the digest, while it is totally absent in the remaining digests. The 21K doublet can be observed even when the digestion has proceeded
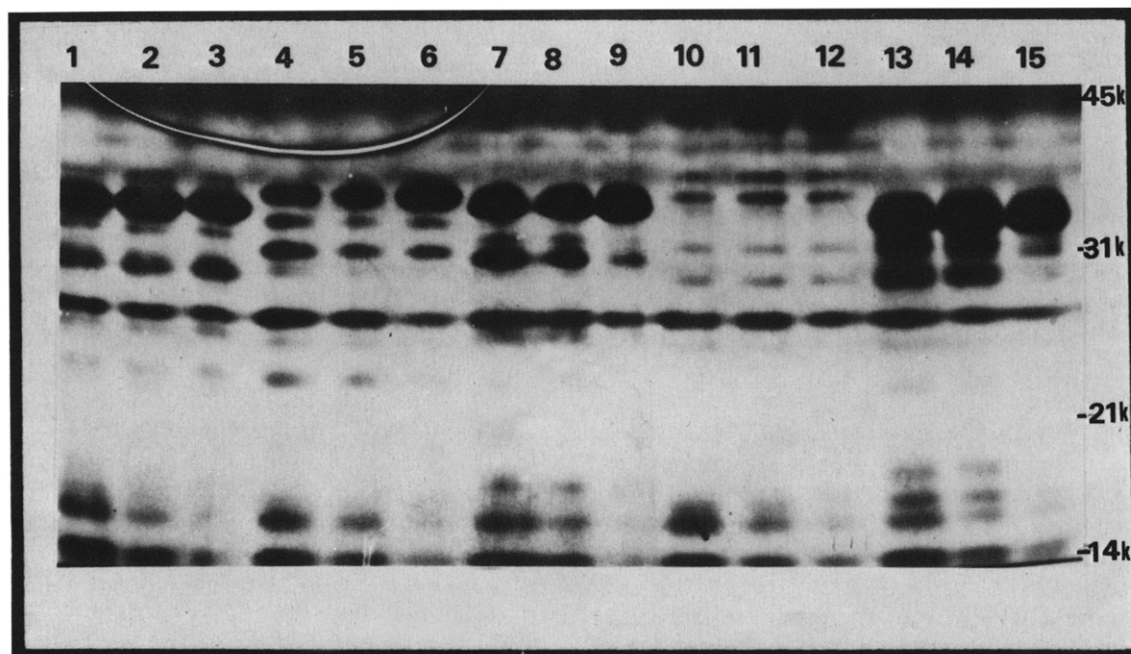

Fig.1. SDS−PAGE analysis (see text for details) of the chymotryptic digestion pattern of tropomyosin components from chicken muscles. Chymotrypsin concentration: 0.1−0.3 μg/digest. Lanes 1−3: α-tropomyosin from leg muscle; lanes 4−6: β-tropomyosin from leg muscle; lanes 7−9: 33K tropomyosin component from gizzard; lanes 10−12: 43K tropomyosin component from gizzard; lanes 13−15: tropomyosin from cardiac muscle. Numbers to the right refer to molecular mass markers: ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme; k, kDa.
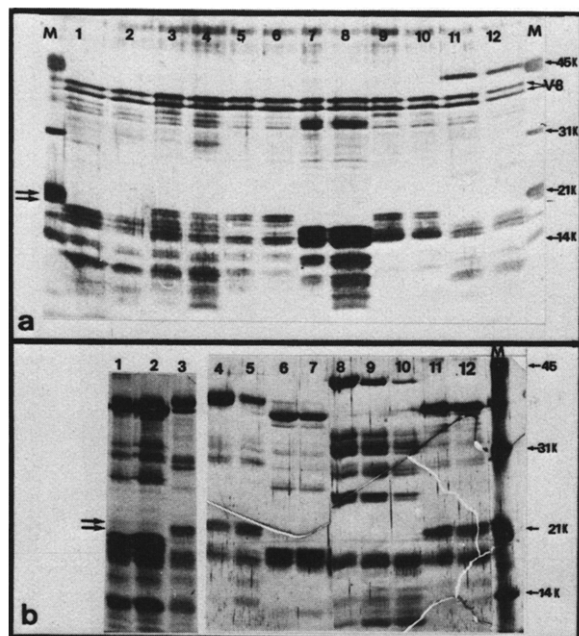
Fig.2. SDS–PAGE analysis (see text for details) of the *Staphylococcus aureus* proteinase (protease-V8) digestion pattern of the tropomyosin components from chicken muscles. (a) High protease concentration (0.1–0.2 µg/digest). Digests analyzed in a 15% acrylamide, 0.1% bis-acrylamide standard gel system. Lanes 1,2: α-tropomyosin from pectoralis major muscle; lanes 3,4: β-tropomyosin from leg muscle; lanes 5,6: β-tropomyosin from PLD muscle; lanes 7,8: tropomyosin component from cardiac muscle; lanes 9,10: 33K component from gizzard; lanes 11,12: 43K component from gizzard; k, kDa. (b) Low protease concentration (0.01–0.001 µg/digest). Digests analyzed in a 12.5% acrylamide, 0.3% bis-acrylamide gel system. Lanes 1,2: α-tropomyosin from PLD muscle; lane 3: β-tropomyosin from ALD muscle; lanes 4,5: β-tropomyosin from PLD muscle; lanes 6,7: tropomyosin component from cardiac muscle; lanes 8–10: 43K component from gizzard; lanes 11,12: 33K component from gizzard; lane M: M_r-markers in the 45–14 kDa range (ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme). Double arrows indicate the position of the 21K doublet.

to completion, at high protease concentration (fig.2a), while it does not appear even transiently, in the remaining digests, even at low protease concentrations (fig.2b). The chicken tropomyosins which produce the 21K doublet are the β-components from ALD, PLD and leg muscles and

the 33K component from gizzard tropomyosin. The doublet is totally absent in the digests from the α-tropomyosin component from pectoralis major, PLD, ALD and leg muscles, the cardiac tropomyosin and the 43K component from gizzard. Chicken cardiac tropomyosin therefore, although it appears to be coded by a messenger RNA which is quite different from the one coding for α-tropomyosin from pectoralis [14], can be classified as an α-like tropomyosin component.

Rabbit skeletal muscle tropomyosin components have a slightly different electrophoretic mobility from their chicken muscle counterparts in the presence or in the absence of urea (not shown). The α-tropomyosin from pectoralis major muscle of the chicken differs in only 11 amino acid positions from the α-tropomyosin from rabbit [15]. The β-tropomyosin from rabbit skeletal muscle has been sequenced [5] but the sequence of β-tropomyosin from chicken remains unknown. Partial-peptide mapping using protease-V8 shows that there is a great degree of similarity between the two tropomyosins (fig.3, lane 1–3; fig.2b, lane 4,5). The β-tropomyosin component from rabbit produces the 21K doublet which is absent in the digests from α-tropomyosin from rabbit (fig.3, lane 10,11). The 21K doublet is also absent in the protease-V8 digests from a non-muscle tropomyosin, derived from pig platelets, which we chose as a representative for the other category of tropomyosin variants that has been described so far (fig.3, lane 5–8).

Two-dimensional analysis of the protease-V8 digests of tropomyosins from chicken and from rabbit shows that the 21K doublet resolves, in all cases, into two peptides which are more basic than either the intact tropomyosin from which they derive, or the remaining peptides, in either the β-like or the α-like digests (fig.4). Their apparent molecular mass would suggest that they contain about 60% of the β-tropomyosin sequence. Considering the cases where a simpler pattern of digestion is obtained, as is the case for β-tropomyosin from rabbit (fig.4h) and the 33K component from gizzard (fig.4e), it can be seen that the two 21K 'basic' peptides 1 and 2, could, in combination with the smaller 'acidic' peptides 3 and 4, respectively, reconstitute the intact tropomyosin molecule. This would therefore suggest that the 21K peptides cover about 60% of tropomyosin,
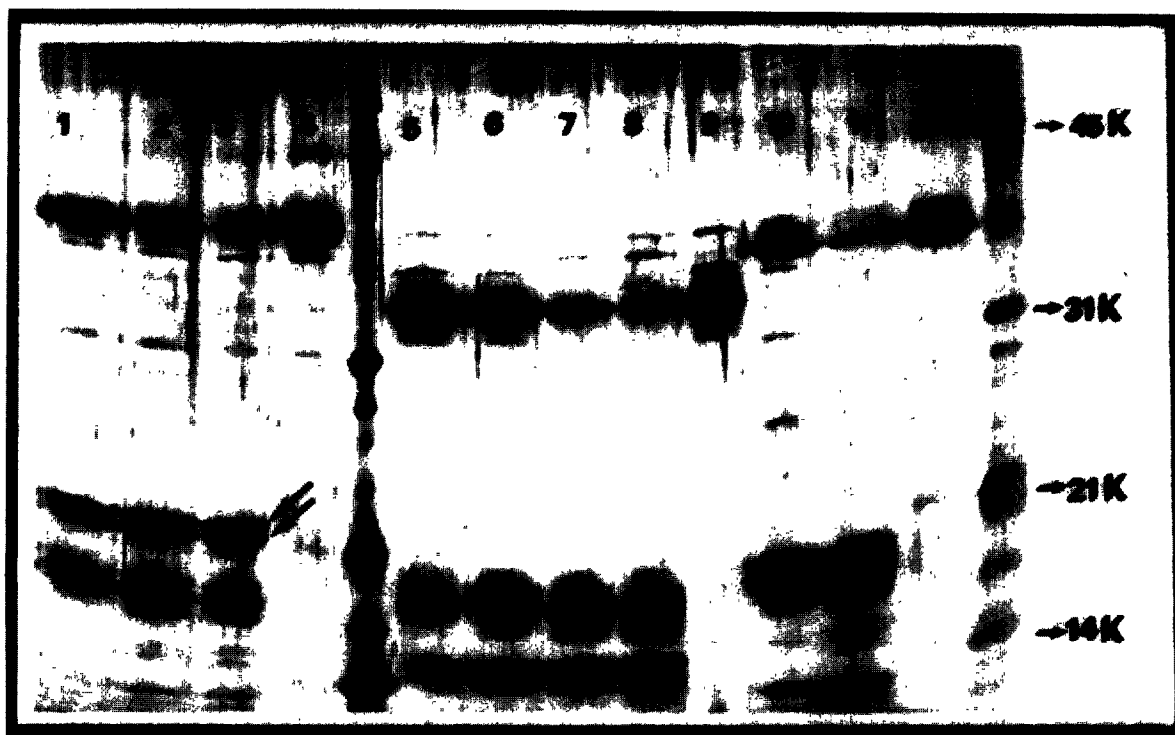
Fig.3. SDS—PAGE analysis (see text for details) of the *Staphylococcus aureus* protease-V8 digestion pattern of rabbit skeletal and pig platelet tropomyosins. Protease concentrations: 0.05–0.001 μg/digest. Lanes 1–3: digests of β-tropomyosin from rabbit skeletal muscle; lane 4: intact rabbit β-tropomyosin; lanes 5–8: pig platelet tropomyosin digests; lane 9: intact pig platelet tropomyosin; lanes 10–12: digests from α-tropomyosin from rabbit skeletal muscle; lane 12: intact α-tropomyosin from rabbit; lane M: M_r-markers as in fig.2. The double arrows indicate the position of the 21K doublet. K, kDa.

starting from one molecular end. Since the sequence of β-tropomyosin from rabbit is known [5], we have looked at the average charge distribution along the molecule. There is an excess of negatively charged amino acids (Glu, Asp), over positively charged amino acids (Arg, Lys) in the whole of the β-tropomyosin sequence. If we define as δ_{R-} the average excess negative charge per residue, i.e.:

δ_{R-} = (number of Glu and Asp residues –
     number of Lys and Arg residues)/
     (total number of amino acid residues)

then we have calculated that δ_R = 0.10 for the intact tropomyosin, $\delta_{R-}(1/2)$ = 0.08 for the -NH$_2$ half of the molecule and $\delta_{R-}(1/2)$ = 0.12 for the -COOH half. This would imply that the -NH$_2$ half of the molecule, being less acidic than the -COOH half, is responsible for the migration of the 21K

peptides in the more basic region of the 2-D gel. Allowing for the fact that the 21K peptides contain more than half of the β-tropomyosin sequence, we have calculated that this 'charge asymmetry' still persists between any large peptides which may contain either the -NH$_2$ or the -COOH ends of tropomyosin.

Our results show that the 21K doublet reflects a structural characteristic of a certain group of tropomyosins. Its absence or presence in the protease-V8 digests of tropomyosins can therefore be employed as a criterion for the classification of various tropomyosins in an α-like or β-like family, respectively, based on a distinct sequence property rather than relying on the relative migration in SDS—PAGE. This is particularly pertinent in the case of chicken gizzard tropomyosin, where the faster migrating component is a β-like
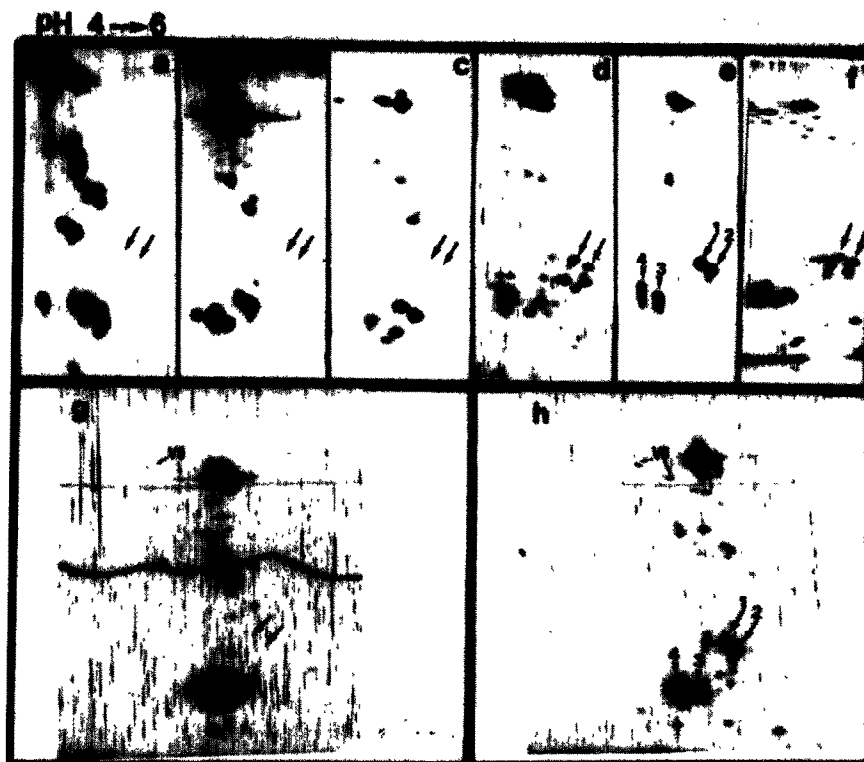
Fig.4. Two-dimensional analysis of the protease-V8 tropomyosin digests. An ampholine range of pH 4–6 was employed for the first dimension. The pH increases from left to right in the second dimension gels (see text for details). (a) 43K Tropomyosin component from gizzard; (b) tropomyosin component from chicken cardiac muscle; (c) α-tropomyosin from chicken PLD muscle; (d) β-tropomyosin from chicken PLD muscle; (e) 33K tropomyosin component from gizzard, digested with a low concentration of protease-V8 (0.001 g); (f) 33K tropomyosin component from gizzard, digested with a high concentration of protease-V8 (0.05 g); (g) α-tropomyosin from rabbit skeletal muscle; (h) β-tropomyosin from rabbit skeletal muscle. -V8- denotes the position of the protease-V8 which can be used as a marker for the comparison of the patterns. Double arrows indicate the position of the two 21K peptides. Arrows 1 and 2 denote the position of the 'basic' peptides and arrowheads 3 and 4 the position of the 'acidic' peptides in the β-tropomyosin digests.

tropomyosin and the slower migrating component is an α-like tropomyosin. Immunochemical evidence renders further support to the latter statement since antibodies raised against α-tropomyosin from pectoralis major muscle and against β-tropomyosin from chicken leg muscle, recognize preferentially the 43K and the 33K gizzard tropomyosin components, respectively (a detailed report to be presented elsewhere). The use of the partial-peptide mapping classification procedure would facilitate the characterization of the tropomyosin components I and II from *Drosophila*, the relative mobility of which changes in the presence of urea [16], as well as the several

non-muscle variants reported [17] and any as yet uncharacterized tropomyosin.

If our assumptions are correct and the 21K peptides contain the -NH$_2$ half of the molecule of β-tropomyosin, this would imply that the α- and β-tropomyosins have certain structural differences halfway along the molecule, which may not be immediately evident when comparing their sequences, but which may be important for their functions. A more basic -NH$_2$ portion of the molecule may result in differences in the properties of some of the actin sites, while a more acidic -COOH portion could influence the troponin-T binding properties of tropomyosin [18]. Finally

this more pronounced 'charge-asymmetry', existing between the two halves of β-tropomyosin, by comparison to α-tropomyosin, may be the reason for the preferential formation of $\alpha\alpha$ or $\alpha\beta$ dimers in a mixed tropomyosin population [12], by inducing the $\beta\beta$ dimer to be less stable.

ACKNOWLEDGEMENTS

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