IDENTIFICATION OF A SECOND BINDING REGION ON RABBIT SKELETAL TROPONIN-T FOR α-TROPOMYOSIN

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1. Introduction

Recent evidence has indicated that a highly helical region (fragment CB2; residues 71–151) of rabbit skeletal muscle troponin-T (Tn-T) is involved in the binding of the troponin complex close to or at the COOH-terminal end (residues 258–284) of the α-tropomyosin (TM) molecule [1,2]. Since the bulk of the troponin complex appears to be located ~1/3rd of the distance from the COOH-terminal end of TM near Cys-190 (summary in [3]), and since the COOH- and NH_2-terminal regions of Tn-T are involved in interactions with Tn-I and Tn-C [4–7], it may be concluded that the Tn-T molecule spans a distance of over 10 nm on the TM–F-actin assembly in the muscle thin filament. Evidence is now available that a second region on the Tn-T molecule may interact with TM. From a chymotryptic digest of Tn-T, fragments T1 and T2 were isolated [6] and were tentatively identified as residues 1–158 and 159–259, respectively [7], both of which were bound to TM immobilized on a Sepharose affinity column. A third fragment, designated herein as T2′ and presumably produced by further proteolytic degradation of T2, showed no interaction with immobilized TM.

Here we confirm the identification of T1 and T2 and the fact that both fragments are bound to TM. T2′, which does not bind to TM, is identified as residues 159–227. Thus the binding of T2 to TM is lost by removing residues 228–259. A fragment B2 (residues 206–258), produced by cleavage at the 2 tryptophan residues of Tn-T [4] is shown to bind to TM, although more weakly than T2. We conclude that a second binding region of TM is present in the COOH-terminal portion of Tn-T involving residues 197–259. Interestingly this segment has a repeating pattern of non-polar residues typical of a coiled-coil [8]. Since it also binds to Tn-C, it is likely that this part of the Tn-T molecule interacts with TM ~1/3rd of the distance from its COOH-terminal end.

Abbreviations: Tn-T, troponin-T; Tn-I, troponin-I; Tn-C, troponin-C; TM, α-tropomyosin; SDS, sodium dodecylsulphate; BNPS–skatole, 2-(2-nitrophenylsulfonyl)-3-methyl-3′-bromoindolenine; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N*′-tetraacetic acid; DTT, dithiothreitol

2. Experimental

2.1. *Chymotryptic digestion of Tn-T and preparation of fragments*

Rabbit skeletal muscle Tn-T, isolated as in [9], was dissolved in 5% formic acid (1.8 mg/ml) and dialyzed against the digestion buffer (50 mM NH_4HCO_3, 0.4 M NaCl, 2 mM $MgCl_2$, pH 8.0). The Tn-T solution was clarified by centrifugation and the Tn-T concentration (40 nmol/ml) determined by amino acid analysis [9]. Digestion with TLCK–α-chymotrypsin (Sigma) was for 15 min at 0°C, using an enzyme/substrate molar ratio of 1:275. The reaction was stopped by heating to 96°C for 5 min. An aliquot was removed for examination on a SDS–urea polyacrylamide gel [10] and the remainder applied to a Sephadex G-75 column (2.0 × 200 cm) equilibrated with the digestion buffer. The peaks, located from their absorbance at 230 nm, were pooled as indicated (fig.1), dialysed and lyophilized. Separation of uncleaved Tn-T from T1 was achieved on a DEAE-cellulose column (1.5 × 20 cm) equilibrated with 1 mM Tris–HCl buffer (pH 8.0) and a gradient from 0 to 0.3 M NaCl (fig.2). The mixture of T2 and T2′ was fractionated on an affinity column of TM immobilized on Sepharose 4B as in [11]. After equilibration of the column with 0.01 M imidazole,

0.1 M NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT (pH 7.0) buffer, the mixture was applied and the column washed with 2 or 3 vol. of the same buffer (fig.3). The bound fragment was subsequently eluted with starting buffer plus 0.5 M LiCl. Peaks obtained by monitoring of the absorbance at 230 nm were pooled, dialyzed, and lyophilized for identification by further analysis.

Fragment B2 (residues 206–258) was obtained by BNPS–skatole cleavage of Tn-T as outlined in [4].

2.2. *Characterization of the chymotryptic fragments of Tn-T*

Details of the procedures involved in amino acid analysis [9], NH_2-terminal dansylation [12], manual Edman degradation [13] and automated sequence analysis [9] have been described.

3. Results and discussion

3.1. *Purification and characterization of chymotryptic fragments*

Application of the chymotryptic digest to gel filtration on Sephadex G-75 (fig.1) resulted in the separation of Tn-T plus T1 from T2 plus T2′ and a low M_r fraction T3. Further purification of T1 was achieved using DEAE-cellulose chromatography (fig.2). A negative result using the dansylation procedure coupled with its amino acid composition (table 1) confirmed the conclusion [7] that T1 spanned residues 1–158.

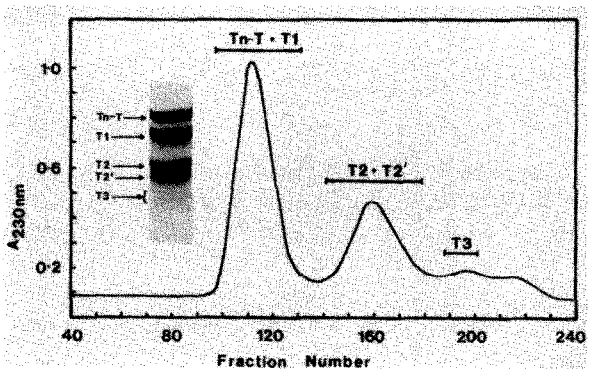

Fig.1. Gel filtration of chymotryptic digest. Tn-T solution (680 nmol in 17 ml) was applied to a Sephadex G-75 column (2 × 200 cm) equilibrated with digestion buffer as in section 2. Fractions (2.5 ml) were monitored at 230 nm. The horizontal bars indicate fractions which were pooled. Inset: SDS–urea 8% gel of digest applied to the column.

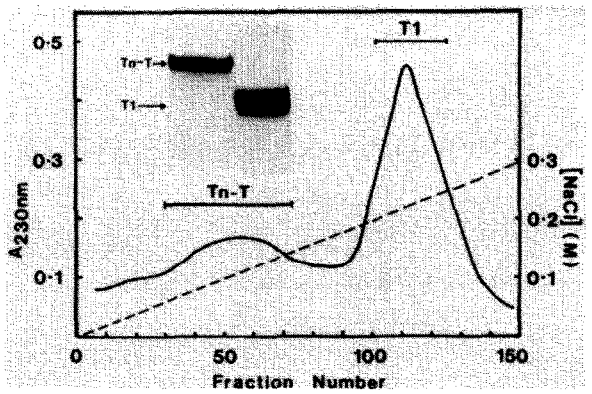

Fig.2. Separation of T1 and Tn-T. The pooled lyophilized mixture of T1 plus Tn-T was taken up in 1 ml starting buffer, any undissolved Tn-T removed by centrifugation and the supernatant applied to a DEAE-cellulose column (1.5 × 20 cm) equilibrated with 1 mM Tris buffer (pH 8.0), linear gradient (– – –), 0–0.3 M NaCl, fraction size, 1.5 ml; absorbance at 230 nm (——). Fractions were pooled as indicated. Inset: SDS–urea 8% gel of purified Tn-T and T1.

Table 1
Amino acid compositions, NH_2-terminal analyses and assigned sequence positions of the chymotryptic fragments from Tn–T[a]

Amino acid	T1	T2	T2′
Asp	11.8 (11)	9.2 (9)	5.6 (7)
Thr	1.0 (1)	4.9 (5)	3.2 (3)
Ser	7.4 (7)	2.6 (2)	1.7 (1)
Glu	46.1 (45)	13.1 (12)	11.3 (11)
Pro	7.1 (8)	0.7 (1)	0.9 (1)
Gly	2.4 (2)	5.8 (6)	2.4 (2)
Ala	16.9 (17)	9.0 (9)	5.5 (5)
Val	8.2 (8)	2.6 (3)	
Met	1.6 (2)	2.4 (3)	0.9 (1)
Ile	4.9 (5)	2.9 (3)	2.0 (2)
Leu	9.0 (9)	9.6 (10)	8.3 (9)
Tyr	1.5 (2)	1.9 (2)	1.8 (2)
Phe	2.0 (2)	2.9 (3)	1.9 (2)
His	4.7 (5)	1.2 (1)	1.0 (1)
Lys	17.5 (18)	19.1 (21)	13.5 (15)
Trp[b]		– (2)	– (1)
Arg	17.0 (16)	8.9 (9)	6.1 (6)
NH_2-terminal analysis	Blocked[c]	L–A–K–[d] (S–S–Y–)[e]	L–A–K–[d] (S–S–Y–)[e]
Assigned position in sequence	1–158	159–259 (156–259)[e]	159–227 (156–227)[e]

[a]Residues/molecule: Integral values in parentheses were determined from sequence analysis [b]Trp was not determined; [c]Dansylation [12]; [d]Automated sequencer analysis [9]; [e]Minor sequencers (<20%)

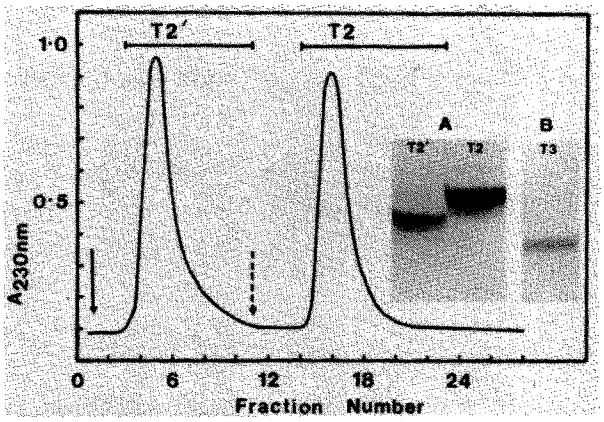

Fig.3. Separation of T2 and T2′ on α-TM–Sepharose affinity column. The pooled lyophilized mixture of T2 and T2′ was dissolved in 0.5 ml of starting buffer and applied to the column (1 × 8 cm) equilibrated with 0.01 M imidazole, 0.1 M NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT (pH 7.0) buffer. Positions of sample application and start of the 0.5 M LiCl buffer are indicated by solid and broken arrows, respectively. The horizontal bars indicate fractions (1.4 ml/tube) which were pooled. Inset: (A) SDS–urea 8% gel of purified T2′ and T2; (B) fragment T3.

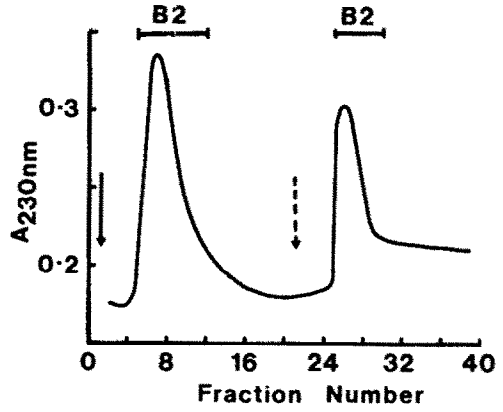

Fig.5. α-TM–Sepharose affinity column profile of fragment B2. Buffer and column conditions are the same as in fig.3. Positions of sample application and start of the 0.5 M LiCl buffer are indicated by solid and broken arrows, respectively.

When fraction T2 plus T2′ was analyzed by dansylation, a major spot of leucine and minor spot of serine was obtained. Subsequent application of the mixture to automated sequencer analysis resulted in one major sequence, Leu–Ala–Lys, and a minor sequence (<20%) Ser–Ser–Tyr. Thus the major fraction of T2 and T2′ begins at residue 159 of Tn-T. Separation of T2 and T2′ was achieved on an α-TM–Sepharose affinity column (fig.3). The T2′ fragment was eluted from the column in the void volume whereas T2 was eluted only after the application of 0.5 M LiCl buffer. NH_2-terminal and amino acid analyses of these fractions (table 1) indicated that the majority of T2 and T2′ fragments corresponded to residues 159–259 and 159–227, respectively. Further evidence for a chymotryptic cleavage of T2 at Tyr-227 to produce T2′ was obtained by the isolation and partial characterization of fragment T3 (fig.1,3). This fragment runs in front of T2′ on SDS–urea polyacrylamide gels and stains only poorly (pink) with Coomassie brilliant blue R250. When the T3 pooled fraction of fig.1 was run on a second Sephadex G-10 column and the void volume peak analyzed by the manual dansyl-Edman procedure, a major sequence of Asx–Ile–Met– was obtained, corresponding to residues 228–230. Although amino acid analyses indicated that the T3 fraction was not completely homogeneous, its overall composition corresponded to residues 228–259 of Tn-T. Upon application to the α-TM–Sepharose affinity column it was eluted in the void volume. These results indicated that the region of T2 responsible for its binding to α-TM was in the COOH-terminal segment of the fragment but that the binding was abolished by cleavage of T2

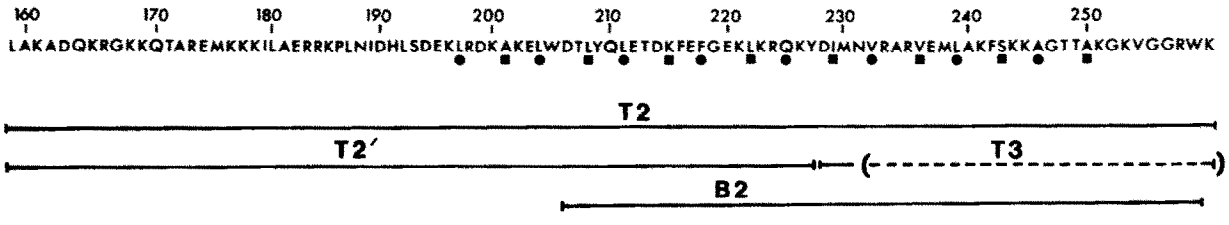

Fig.4. Position of fragments in the sequence of Tn-T. The COOH-terminal sequence of Tn-T is given in one letter code. Top line indicates residue numbers. The heptapeptide repeating pattern of hydrophobic residues from 197–250 [8] is shown in solid circles and squares. Fragment T3 starts at the sequence ^{228}D–I–M–, and is assumed to stretch to 259.

into subfragments T2′ (residues 159–227) and T3 (residues 228–259). The relationship between these fragments is shown in fig.4.

When fragment B2 (residues 206–258), prepared by BNPS–skatole cleavage of the tryptophans of Tn-T, was applied to the α-TM–Sepharose affinity column, it was partially eluted in the void volume while the remainder was eluted with 0.5 M LiCl (fig.5). Fragment B2 is 22 residues larger than T3. The differences observed in the degree of binding of the 3 fragments (T2 > B2 > T3) suggest that the binding region extends over a relatively large portion of the COOH-terminus of Tn-T. A heptad hydrophobic repeat exists in Tn-T from residues 197–250 (fig.4) [8] similar to that in TM and typical of a coiled-coil structure [14]. Since this region encompasses that part of the Tn-T molecule implicated here as binding to α-TM, it is possible that the interaction between residues 197–250 of Tn-T and α-TM involves the formation of a triple-stranded coiled-coil or its interaction with one of the two strands of α-TM to form a two-stranded coiled-coil. Since this same region of Tn-T (residues 159–259) also interacts with Tn-C [4,6] it is highly likely that the part of the α-TM structure with which this second binding segment of Tn-T interacts is in the vicinity of Cys-190 or ~1/3rd of the distance from the COOH-terminal end of TM. That this is the case is indicated in [15] in which the fluorescent probe *N*-(1-anilino-napthyl-4)-maleimide was attached to Cys-190 of α-TM and the effects of the addition of fragments T1 and T2 tested. While T1 had no effect on the fluorescence of the label, the presence of T2 caused an enhancement. Further studies designed to investigate the binding of these two regions of the Tn-T molecule on the structure of TM are presently in progress.

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