STABILITY AND TROPONIN-T BINDING PROPERTIES OF RABBIT SKELETAL α-TROPOMYOSIN FRAGMENTS

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1. Introduction

Contraction in vertebrate skeletal muscle is regulated by the thin filament proteins, tropomyosin and the troponin complex. A fuller understanding of the mechanism of this process requires more information on the conformational properties of these proteins and the nature of the interactions between them. In this study fragments of α-tropomyosin were prepared by selective proteolytic and chemical methods and characterized with respect to their helical contents and thermal stability. The various fragments were also compared with respect to their relative binding affinities to troponin immobilized on Sepharose columns. The results show that two regions of the molecule (residues 13–125 and residues 183–244) are relatively resistant to prolonged tryptic digestion. Although fragments derived from the COOH-terminal half of the molecule have less helical content and a lower thermal stability than fragments derived from the NH_2-terminal half, the former bind significantly more strongly to immobilized troponin and troponin-T. However all fragments, including non-polymerizable α-tropomyosin prepared by treatment with carboxypeptidase, bind more weakly than does intact α-tropomyosin.

2. Experimental

Tropomyosin was prepared from rabbit skeletal muscle and fractionated into its α- and β-components [1]. Short-term tryptic digestion of α-tropomyosin was performed at 0°C in 50 mM NH_4HCO_3 buffer, pH 8.5, for 1 min at enzyme/substrate ratio 1:200. A long-term digestion was done at 0°C in 0.4 M NaCl, 0.1 M Tris buffer, pH 8.5 for 24 h at enzyme/protein ratio 1:50. The reactions were stopped by either lowering the pH to 2 or by the addition of lima bean trypsin inhibitor. Cyanogen bromide fragments Cn1A and Cn1B were prepared as in [2,3]. Specific cleavage at cysteine-190 [4] was used to produce fragments Cy1 and Cy2. Fragments were separated from uncleaved tropomyosin and from each other by gel filtration on Sephadex G-75 in 10% formic acid followed by ion-exchange chromatography on CM-cellulose at pH 4.0 and/or on QAE-Sephadex, at pH 7.5, under the same conditions as in [1–3]. The identity and purity of all fragments was established by SDS–gel electrophoresis, amino acid, NH_2-terminal and COOH-terminal analyses. Non-polymerizable α-tropomyosin was prepared by digestion with carboxypeptidase A for 6 h as in [5] except that the enzyme/substrate ratio was 1:100. Under these conditions 7–8 residues are removed from the COOH-terminus. The helical content and melting temperature (T_m) of each fragment in 0.1 M KCl, 1 mM DTT, 50 mM sodium phosphate buffer, pH 7.0, was determined on a Cary 6001 circular dichroism attachment to a Cary 60 recording spectropolarimeter. Troponin-T was coupled to CNBr-activated Sepharose 4B by standard procedures. The resulting material was packed in a 0.9 × 8 cm column and equilibrated with the starting buffer: 1 mM DTT; 1 mM EGTA; 10 mM imidazole buffer, pH 7.0, 4°C. After application of the sample, at least 2 column vol. starting buffer were passed through the column before a salt gradient of 0–0.5 KCl was established.

3. Results

The conditions chosen for the enzymatic digestions resulted from studies of the time course of tryptic action on α-tropomyosin as monitored by SDS–urea polyacrylamide gel electrophoresis. These indicated that by exposing the protein for very brief periods to the action of the protease, fragments could be produced which resulted from a single peptide bond cleavage. After brief tryptic digestion two fragments (T1 and T2) were obtained which resulted from an initial cleavage at arginine-133. Further digestion resulted in the degradation of these to smaller fragments some of which however were relatively resistant to prolonged tryptic digestion. Thus it was possible after 24 h tryptic digestion to isolate fragments T3 (residues 13–125), T4 (residues 183–284) and T5 (residues 183–244).

A comparison of the helical contents and thermal stabilities of the various fragments derived from tryptic digestion as well as from specific chemical cleavage at methionine and cysteine residues is presented in table 1. These data show that all the fragments, with the exception of T5 (residues 183–244) which is the smallest, have a relatively high percentage of α-helical content. It is also clear that fragments derived from the COOH-terminal end of the molecule have a lower helical content and a lower heat stability as estimated from their T_m values than do the fragments derived from the NH_2-terminal end.

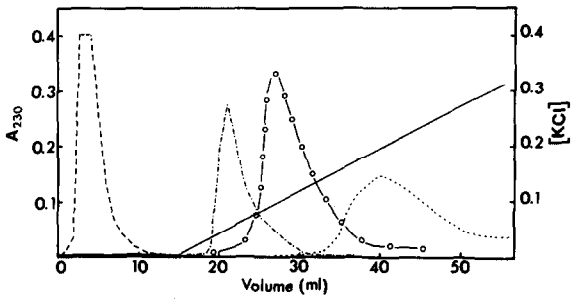

Fig.1. Elution profiles of bovine serum albumin (– – –), Cn1A (– · – · –), Cn1B (–o–o–) and α-tropomyosin (· · · · ·) on a troponin-T Sepharose affinity column (0.9 × 8 cm). After application of the sample the column was eluted with at least 2 column vol. starting buffer (see section 2) before establishing a gradient of 0–0.5 M KCl (——).

The results of experiments using a troponin-T affinity column to test the relative binding of the various fragments to the tropomyosin-binding component of troponin are shown in fig.1 and table 1.

Table 1
Helical contents, thermal stabilities and binding to troponin-T–Sepharose of α-tropomyosin and its fragments

	Residue no.	Helical content (%)[a] at 10°C	T_m (°C)	Average α-parameter[b]	Elution [KCl] (M)
α-Tropomyosin	1–284	92	44	1.185	0.20
NH_2-Terminal fragments					
T1	1–133	89	49	1.194	0.05
T3	13–125	89	47	1.218	0.05
Cn1A	11–127	81	47	1.202	0.05
Cy1	1–189	63	48	1.197	0.05
COOH-Terminal fragments					
T2	134–284	81	39	1.176	0.10
T4	183–284	64	33	1.160	0.10
T5	183–244	17	–	1.199	–
Cn1B	142–281	72	29	1.130	0.10
Cy2	190–284	62	28	1.143	0.10
Non-polymerizable tropomyosin	1–276	–	–	–	0.10

[a] Calculated from the mean residue ellipticity at 222 nm [6,16,17]
[b] Calculated from the amino acid composition and values in [14]

Figure 1 shows representative elution profiles of the cyanogen bromide fragments, Cn1A and Cn1B, bovine serum albumin and α-tropomyosin from the troponin-T affinity column. Similar results were obtained when either whole troponin or a cyanogen bromide fragment of troponin-T, CB1, known to bind to α-tropomyosin [6,7], were used to prepare the affinity column. Bovine serum albumin is not retarded by the column, Cn1A is eluted at a salt concentration of 0.05 M, Cn1B at 0.10 M and α-tropomyosin at 0.20 M. The salt concentrations required for elution of the other fragments are given in table 1. These results show that although none of the fragments bind as strongly to troponin-T as does intact α-tropomyosin, there is a significantly higher binding affinity of fragments derived from the COOH-terminal half of the molecule than those derived from the NH_2-terminal half. The binding of the NH_2-terminal fragments is probably largely non-specific in nature, possibly dependent on the charge properties and structure of coiled-coils, since myosin subfragment 2 as well as cyanogen bromide fragment(s) of light meromyosin were eluted at the same ionic strength.

The possible importance of the COOH-terminal and NH_2-terminal overlap regions of the tropomyosin molecule in troponin binding was also examined by testing the binding of non-polymerizable α-tropomyosin prepared by treatment with carboxypeptidase A. As indicated in table 1, this product was eluted at a significantly lower salt concentration (0.10 M) than intact α-tropomyosin. Since none of the fragments of course contain both the necessary NH_2-terminal and COOH-terminal sequences implicated in head-to-tail aggregation, it is not surprising then that they all bind considerably less strongly than intact α-tropomyosin.

4. Discussion

The initial site of cleavage by trypsin at peptide bond 133–134 would indicate that this region of the structure is less ordered than the remainder of the coiled-coil of α-tropomyosin. It is probably significant that this site is close to aspartate-137 which is the only such residue to occur in a 'core' position in the structure of this protein [3,8–10]. The isolation of fragments T3 (residues 13–125), T4 (residues 183–284) and T5 (residues 183–244) from a long-term tryptic digestion shows that these regions of the structure are relatively resistant to tryptic attack at 0°C. Similar trypsin-resistant fragments have been described [11–13] but in only one case have they been tentatively assigned in the amino acid sequence [13].

The higher helical contents and thermal stabilities of the fragments from the NH_2-terminal half of the molecule as compared with those from the COOH-terminal appear to correlate with their relative contents of helix formers and breakers as defined by the criteria in [14]. This is illustrated in table 1 where the average helical parameters have been calculated for each of the fragments. This is particularly true of the two cyanogen bromide fragments Cn1A and Cn1B where the large difference in thermal stability can be related to the higher content in Cn1B of such poor helical formers as tyrosine, isoleucine and serine.

The observation that the COOH-terminal fragments of α-tropomyosin are eluted at a higher salt concentration from the immobilized troponin-T Sepharose column than are fragments derived from the NH_2-terminal half is consistent with the hypothesis that the troponin-T binding site is positioned about 1/3 the distance from the COOH-terminal end of the molecule. For a summary of the evidence on which this hypothesis is based see [15]. In the case of the carboxypeptidase treated tropomyosin which is eluted at the same ionic strength as the COOH-terminal fragments (0.10 M) and at 1/2 the ionic strength required for the elution of intact α-tropomyosin (0.20 M), the results are explicable in terms of the inability of this material to polymerize into long filaments by head-to-tail aggregation. Polymerized tropomyosin with its multiple troponin-T attachment sites would be expected to bind more strongly to the immobilized troponin-T than non-polymerizable tropomyosin. Thus, based on the present experimental evidence it is not necessary to invoke a more direct role for the COOH-terminus of tropomyosin in the binding of troponin.

However, the possible importance of an indirect role of the head-to-tail overlap of tropomyosin molecules in the binding of troponin is indicated by the remarkable viscosity increases that occur when troponin, troponin-T and certain Tn-T fragments are complexed with tropomyosin in solution [18–20] and which are abolished with non-polymerizable

tropomyosin [21]. If one accepts that the binding site for troponin-T on tropomyosin is centered about 1/3 from the COOH-terminus, then this phenomenon can only be understood in terms of a conformational change induced by the binding of troponin and transmitted to the head-to-tail overlap along the length of the coiled-coil structure. If such changes in the region of the putative troponin binding site can affect the extent of head-to-tail aggregation of tropomyosin molecules, then the reverse phenomenon, namely, an effect of the degree of polymerization on the binding affinity of tropomyosin for troponin can be anticipated, a result which is consistent with the results of this study.

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