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NON-POLYMERIZABLE TROPOMYOSIN: PREPARATION, SOME PROPERTIES AND F-ACTIN BINDING

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SUMMARY: Conditions are described for the nearly quantitative removal of residues 274-284 from the COOH-terminus of rabbit cardiac tropomyosin. The product is monomeric (Mr = 64,000) even at low ionic strength. The circular dichroism melting profile is only marginally different from the intact protein. The failure of the non-polymerizable tropomyosin to bind to F-actin, even at elevated $MgCl_2$ concentrations, illustrates the importance of the head-to-tail interaction of tropomyosin molecules in their cooperative binding to the thin filament structure.

Accumulating evidence indicates that the head-to-tail polymerization of tropomyosin (TM)[+] molecules, involving an 8 to 9 amino acid residue overlap between the COOH- and NH_2-terminal ends of adjacent molecules, is of crucial importance for the assembly and function of the thin filament protein complex of striated muscle. The addition of whole troponin (Tn) complex to a TM solution markedly increases its viscosity, a phenomenon that is mimicked by a soluble fragment, CB1 (residues 1-151), of troponin-T (1-3). Recent observations indicate that CB1 induces head-to-tail polymerization of TM fragments in which the NH_2-terminal and COOH-terminal sequences of the original TM molecule are intact (4) and that this occurs through the 'binding' of CB1 close to or at the head-to-tail overlap region (5). Consistent with this interpretation, Côté et al. (6,7) observed that equine platelet TM, which has markedly different NH_2- and COOH-terminal sequences from its muscle counterpart,

[+]Abbreviations: TM, tropomyosin; Tn, troponin; NPTM, non-polymerizable tropomyosin; DFP, diisopropylfluorophosphate; SDS, sodium dodecylsulfate; DTT, dithiothreitol.

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aggregates poorly in a head-to-tail manner and binds only weakly to CB1. Further, recent studies suggest that the cooperative binding of TM to F-actin (8-10) and of myosin subfragment-1 to regulated F-actin (11,12) are, at least in part, mediated by interactions between adjacent TM molecules along the thin filament structure. The demonstration that serine-283, the penultimate residue at the COOH-terminus of TM, exists partly in a phosphorylated form (13) also emphasizes the importance of this region as an important structural element in the assembly and function of the thin filament protein complex.

To facilitate additional investigations of the role of head-to-tail polymerization of TM molecules in thin filament assembly and regulation, a discrete species of non-polymerizable TM (NPTM) would be of considerable value. Although such a product has been previously prepared (14,15) by the removal of several COOH-terminal residues with carboxypeptidase A, these preparations of NPTM undoubtedly contained a heterogeneous population of molecules differing in the number of residues removed by the action of the enzyme. In this paper we report essentially quantitative removal (>90%) of 11 residues from the COOH-terminus of rabbit cardiac TM. The product, NPTM, is monomeric even at low ionic strengths and its temperature stability is only marginally different from intact TM. It no longer binds to F-actin even at high concentrations of $MgCl_2$.

MATERIALS AND METHODS

Proteins - Cardiac TM was prepared from rabbit hearts (Pelfreez) as described (16) and purified on hydroxylapatite (17). Published procedures (18,19) were used for the preparation of G-actin and Tn from rabbit skeletal muscle.

Preparation of NPTM - Commercially available diisopropylfluorophosphate (DFP)-treated carboxypeptidase A (Pancreas, Sigma) always contains traces of endopeptidase activities. Therefore, a stock solution of the enzyme (7.9 mg/ml) in 2 M NH_4HCO_3, pH 8.0 was prepared and treated with fresh DFP according to the method of Ambler (20) and Potts (21). Cardiac TM (5 mg/ml) in 10 mM Tris-HCl, 0.1 M KCl, 0.01% sodium azide, pH 8.0 was incubated at 37°C. and DFP-treated enzyme added to a final enzyme to substrate weight ratio of 1:50. The course of digestion was followed by taking aliquots for amino acid analyses (see Fig. 1) and sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis. For the former, 100 ul of the reaction mixture was added to 15 ul of 88% formic acid, freeze-dried and dissolved directly in the pH 2.2 sodium citrate buffer for analysis on a Durrum D500 analyser. No precautions were taken to remove protein which was eluted just before histidine. For gel electrophoresis, 20 ul of the reaction mixture was added to 100 ul of SDS-urea buffer (50 mM sodium

phosphate, 1% SDS, 6 M urea, pH 7.0), heated at 96°C for 3 min and analyzed on a 15% polyacrylamide gel as described by Laemmli (22).

For bulk preparation of NPTM, the digestion was stopped at the optimum time (normally 6 h) by heating to 85°C for 3 min to denature the carboxypeptidase A and any endopeptidase contamination. Control experiments have shown that this treatment has no effect on the α-helical contents or temperature transition profiles of α-TM and NPTM as assessed by circular dichroism (CD). The pH was lowered to 7.0 with 1 M HCl and the solution centrifuged to remove the precipitated carboxypeptidase A. The supernatant was adjusted to pH 2.0 and dialyzed exhaustively against 1% formic acid at 4°C and freeze dried.

CD and sedimentation equilibrium ultracentrifugation - Melting curves and α-helical contents of cardiac TM and NPTM in 0.1 M KCl, 50 mM sodium phosphate, 0.01% sodium azide, 2 mM dithiothreitol (DTT), pH 7.0 were measured as described (23). All samples were initially reduced with 50 mM DTT and dialyzed against the analysis buffer under nitrogen. Sedimentation equilibrium ultracentrifugation was performed at 20°C on a Beckman Spinco Model E analytical ultracentrifuge based on the methodology of Richards et al. (24). The experiments were carried out at different ionic strengths, 10 mM, 0.1 M and 1.0 M KCl, in 10 mM sodium phosphate, 1 mM DTT, pH 7.0.

Tropomyosin-actin binding studies - These were based on the methods of Eaton et al. (25) in a buffer containing 3 mM imidazole, 0.1 M KCl, 5 mM $MgCl_2$, 2 mM ATP, 1 mM EGTA, 2 mM DTT, pH 7.0. The final concentration of F-actin was 10 uM and the total TM (or NPTM) concentration varied from 0 to 5 uM. Centrifugation was carried out at 20°C for 90 min at 100,000 g.

RESULTS AND DISCUSSION

Time course of release of amino acids - The order of release of amino acids was in essential agreement with the known amino acid sequence (Fig. 1). After 6 h of digestion, over 90% of residues 273-284 was released from the COOH-terminus of TM. Low recoveries of serine and asparagine were obtained as explained elsewhere (5). Glutamic acid was never released in amounts greater than 0.1 mole/mole of polypeptide chain in agreement with the known specificity of the enzyme which is ineffective towards two or more acidic residues occupying adjacent positions (20). After recovery of the product, over 90% of the protein migrated on SDS polyacrylamide gel electrophoresis with a slightly greater mobility than intact TM, in agreement with the amino acid analyses. The heat treatment provided a convenient way of denaturing and quantitatively removing the carboxypeptidase and perhaps any contaminating endopeptidase (see Fig. 2).

Circular dichroism and sedimentation equilibrium ultracentrifugation studies - That the overall stability of the coiled-coil structure of α-TM was not significantly affected by the enzymic treatment was shown from estimates of the

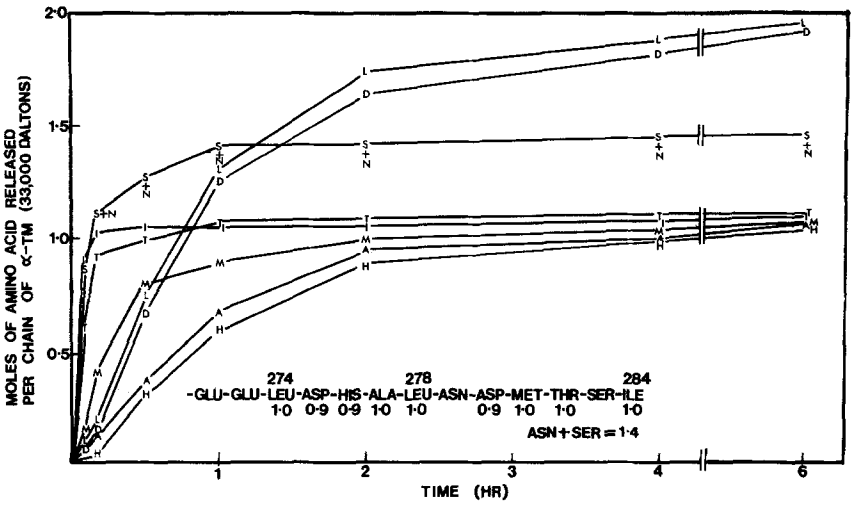

Figure 1. Time course of amino acids released from α-TM by carboxypeptidase A at 37°C. The weight ratio of enzyme to substrate was 1:50. The release of each amino acid is shown by -x-, where x represents the one-letter code of the amino acid: L = Leu, D = Asp, S = Ser, N = Asn, T = Thr, I = Ile, M = Met, A = Ala and H = His. Inset shows the COOH-terminal sequence of α-TM and the number below each residue represents the moles of amino acid released per mole of polypeptide chain (33,000 daltons) after 6 h of digestion.

α-helical contents of NPTM and α-TM as a function of temperature from circular dichroism measurements (Fig. 3). However, some destabilization was indicated by the slightly lower α-helical content at 10°C of NPTM (92 ± 2%; average of 3 determinations) as compared with α-TM (96 ± 2%; average of 3 determinations) and a small decrease in the temperature (Tm) at which 50% of the structure was lost in a thermal denaturation profile (Fig. 3). The Tm for NPTM was 42 ± 1°C as compared with 44 ± 2°C for α-Tm; averages of 2 determinations. A comparison of these profiles (Fig. 3) for NPTM and TM indicates that the differences largely occur at temperatures below the Tm and can probably be attributed to a local destabilization of the structure at the COOH-terminal end. Such an interpretation is consistent with the known relative instability of the COOH-terminal half of the TM molecule as compared with the NH_2-terminal half (23, 26-28) and with our earlier observation (5) that the extent of iodination by ^{125}I and lactoperoxidase of tyrosine-267 in NPTM is almost twice that in TM.

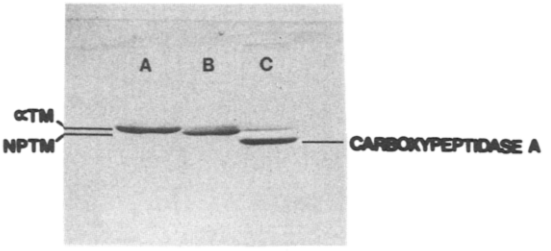

Figure 2. **SDS polyacrylamide gel electrophoretic patterns of: A.** cardiac α-TM; B. NPTM from pH 7.0 supernatant after heat treatment of carboxypeptidase A digestion (6 hr); C. pH 7.0 precipitate after heat treatment of carboxypeptidase A digestion (6 hr).

That NPTM has lost its ability to polymerize in a head-to-tail manner was demonstrated by analytical sedimentation equilibrium measurements of its molecular weight in buffers of low ionic strength at neutral pH. Even in 10 mM KCl, 10 mM sodium phosphate, pH 7.0, conditions under which native α-TM shows extensive aggregation (30), NPTM was found to have a molecular weight of 64,000.

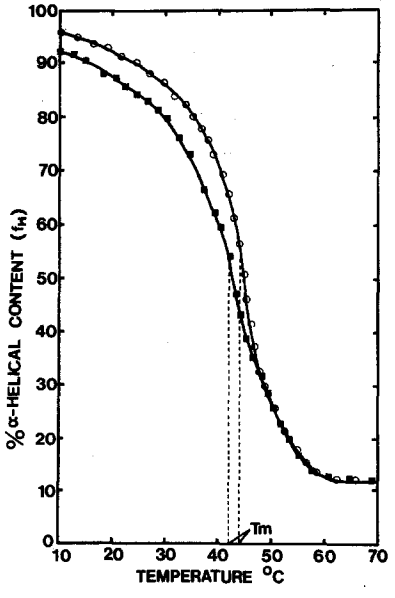

Figure 3. Melting transition profiles of cardiac TM and NPTM. The α-helical content, f_H, was calculated from the mean residue ellipticity at 222 nm [31]. The Tm was taken as that temperature at which:

$$[f_H]^T = [f_H]^{70°C} + 0.5([f_H]^{10°C} - [f_H]^{70°C})$$

Non-polymerizable TM, —■—■—; cardiac TM, —○—○—.

Lack of binding of NPTM to F-actin - Previous studies (8-10) have demonstrated the highly cooperative binding of TM to F-actin at a molar ratio of 1:7 (TM: F-actin monomer). Under similar conditions (0.1 M KCl, 5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, 1 mM EGTA, pH 7.0), the binding of NPTM was negligible (<0.07 TM to 7 actin monomers). Binding was not induced by increasing the $MgCl_2$ concentration to 10 mM. These results illustrate in a dramatic way the importance of the head-to-tail overlap region of TM in the assembly of the thin filament structure. These observations are in excellent agreement with the recent analyses of Walsh and Wegner (10) who have shown that the equilibrium constant for binding of TM to contiguous sites on F-actin is 10^3 to 10^4 times higher than that for the binding to isolated sites. This difference results in the highly cooperative binding of TM to F-actin (8-10) and on the basis of the present evidence is undoubtedly attributable to the head-to-tail interaction of TM molecules. Previous observations that NPTM binds to F-actin (14), although perhaps more weakly (15), were with preparations of NPTM that had been treated with carboxypeptidase in a less exhaustive manner and which therefore undoubtedly retained a significant proportion of the head-to-tail overlap region in their structures.

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