Primary Structure of Rabbit Skeletal Muscle Troponin-T

**SEQUENCE DETERMINATION OF THE NH₂-TERMINAL FRAGMENT CB3 AND THE COMPLETE SEQUENCE OF TROPONIN-T**

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The amino acid sequence of CB3, the NH₂-terminal fragment of troponin-T, and the alignment of all six cyanogen bromide (CB) fragments are reported. Fragment CB3, comprised of 70 residues, has eight of the nine prolines of tropomyosin. As observed in other proteins of the myofibrillar system, its NH₂ terminus is blocked by an acetyl group. Methionine-containing "overlap" peptides isolated from a pepsin digest of troponin-T as well as Z-(Z-nitrophenylsulfenyl)-3-methyl-3'-bromoindolenine cleavage of the protein were used to order the fragments as CB3-CB2-CB5-CB4-CB7-CB6. The complete sequence of troponin-T, a single polypeptide chain of 259 amino acids having a molecular weight of 30,500, is presented.

Tropomin-T of rabbit skeletal muscle is the tropomyosin-binding component of the tropontin complex. In the previous two papers of this series (1, 2) the purification of the six cyanogen bromide fragments and the sequence determination of five of these fragments (CB2, CB4, CB5, CB6, and CB7) were described. The present report concerns the elucidation of the final fragment, NH₂-terminal CB3, and the assembly of the complete sequence of Tn-T.

**METHODS**

Purification of Peptides – Column chromatography on Sephadex G-75 and high voltage electrophoresis were carried out as described in the first paper of this series (1). Whenever an enzymatic digest of CB3 was fractionated by the latter method, the resulting ninhydrin-negative blocked NH₂-terminal peptide was found by systematically cutting out 1-inch bands throughout the unainted region of the paper and subjecting the eluted samples to an amino acid analysis. This method was successful in locating several varieties of NH₂-terminal peptides which differed in length.

Enzymic Digestions – Pepsin digestion of peptides was carried out in 5% formic acid for 5 min at 25°C using an enzyme to substrate molar ratio of 1:70.

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1 The abbreviations used are: Tn-T, troponin-T; dansyl, 5-di-methylaminonapthalene-1-sulfonyl; DNPS-skatole, 2-(2-nitrophenylsulfonyl)-3-methyl-3'-bromoindolenine; TLC, thin layer chromatography; PTH, 3-phenyl-2-thiodynantoin.

2 The abbreviations used are: Tn-T, troponin-T; dansyl, 5-di-methylaminonapthalene-1-sulfonyl; DNPS-skatole, 2-(2-nitrophenylsulfonyl)-3-methyl-3'-bromoindolenine; TLC, thin layer chromatography; PTH, 3-phenyl-2-thiodynantoin.

3 Some of the data are presented as a miniprint supplement immediately following this paper. Tables I to V and VII to XIII and Figs. 1, 3, and 6 are found on pp. 988-989. For the convenience of those who prefer to obtain supplementary material in the form of 21 pages of full size photocopies, these same data are available as JBC Document No. 76M-9.15. Orders for supplementary material should specify the title, authors, and references to this paper. The JBC Document Number, and the number of copies desired. Orders should be addressed to the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $3.15.
Sequence of Rabbit Skeletal Troponin-T

Smillie (6) to give three fractions (I, II, III) which were then chromatographed on Technicon Chromobead P ion exchange resin using gradient system I of Wellinder and Smillie (7). Methionine content of the Chromobead P fractions was determined by amino acid analysis after acid hydrolysis, and methionine peptides were then purified from the methionine-containing peaks using paper electrophoresis at pH 6.5 or pH 1.8, or both.

Cleavage of Tn-T by BNPS-skatole—Tn-T (60 mg) was cleaved by the BNPS-skatole method described in the second paper (2), and the extracted lyophilized digest fractionated on a column of G-75 Sephadex (100 x 2.5 cm) in 5% formic acid.

RESULTS AND DISCUSSION

Identification of Phosphoserine in CB3—A phospho analysis of CB3 after complete acid hydrolysis indicated a 1:1 molar ratio of phospho/CB3. Partial acid hydrolysis conditions were investigated to maximize peptide bond cleavage while leaving standard phosphoserine intact. Suitable conditions (6 n HCl, 110°, 2 h) were applied to CB3, and the hydrolyzed sample separated by high voltage paper electrophoresis at pH 6.5 and 1.8. When the eluted band having the same mobility as standard phosphoserine was subjected to amino acid analysis, a symmetrical peak was observed in the position of phosphoserine. When the sample was initially hydrolyzed using 6 n HCl, 110° for 20 h, serine was observed. It was therefore concluded that phosphoserine is present in CB3.

Peptides from Tryptic Digestion of Citraconylated CB3—The amino acid composition of CB3 (1), indicating 7 lysines and 2 arginines, suggested a preliminary tryptic digestion of citraconylated CB3. Separation of the dectaconylated products of this digestion by paper electrophoresis at pH 6.5 and 1.8 yielded only two peptides, T1 and T2, indicating that one of the arginines was not cleaved. T1, which had a blocked NH2 terminus and an amino acid composition consistent with a length of about 64 residues, was recovered in poor yield. T2 was a hexapeptide containing homoserine and an NH2-terminal glutamyl residue. Tryptic digestion of T2 produced two peptides T2-t1 and T2-t2 (Table I). The sequence of T2-t1 was established as follows. Since the NH2 terminus of T2 was a glutamyl residue, and since trypsin is known to be specific for lysine, the order of the amino acids from the composition must be Glu-Asn-Lys. Amidases were assigned to both the glutamyl and asparagyl residues because of the positive net charge of T2-t1 at pH 6.5.

Peptide T1 was recovered quantitatively by fractionating the tryptic digest of citraconylated CB3 on a Sephadex G-75 column (Fig. I). The peptides derived from a tryptic digest of decitraconylated T1 are summarized in Table I. Peptide T1-t3 was further cleaved with pepsin at phenylalanine 57 to give two peptides of suitable length for manual sequencing (Table I). The 43-residue fragment T1-t1, subjected to further digestion with thermolysin, is discussed later.

Peptides from α-Lytic Protease Digestion of T1—The 11 peptides isolated from high voltage paper electrophoresis at pH 6.5 (and 1.8) are summarized in Table II. Peptide T1-AP9 was taken through only 6 Edman cycles to overlap T1-42 and T1-43 (see Fig. 2). Since very little of T1-AP10 was recovered and the sequence of this region was already known from T1-t3, samples were removed only for the NH2-terminal residue and the last 4 steps at the COOH terminus to provide an overlap for peptides T1-45 and T1-44. Glutamine 61 was already assigned in T1-t4.

Partial cleavage at valine 5 produced two species of peptides having blocked NH2 termini: a pentapeptide T1-AP2 and an octapeptide T1-AP1. Both peptides were located by eluting 1-inch ninhydrin-negative bands off the paper after high voltage electrophoresis and subjecting samples to amino acid analyses. In the penicilliocarboxypeptidase-S treatment of T1-AP1 (Table III), cleavage stopped after glutamic acid 3 was released. When a fresh aliquot of enzyme was added to T1-AP1 after 8 h and the digestion allowed to proceed for an additional 16 h, no further release of amino acid occurred, even though penicilliocarboxypeptidase-S can normally cleave between serine and an asparagyl residue. As previously discussed, phosphoserine was isolated from fragment CB3. In a phosphate determination of peptide T1-AP1, 0.8 μmol of phosphate was recovered from 1.0 μmol of peptide. These results were in agreement with an earlier report (8) that the serine near the NH2 terminus is always phosphorylated when troponin-T is isolated according to the method of Ebashi et al. (9). It is therefore conceivable that the phosphate group prevents the peptide bond between serine and the asparagyl residue from being accessible to the enzyme. This explanation seemed quite likely when further studies were carried out on T1-AP1.

Sequence Determination of NH2-terminal Octapeptide T1-AP1—In order to ascertain the order of the serine and asparagyl residue, attempts were made to find conditions that would remove the blocking group but leave the remaining peptide intact. Partial acid hydrolysis for 0.5 to 1 h using 0.03 n HCl at 110° proved to be successful in achieving the desired results. The hydrolyzed sample was dried down and separated by high voltage paper electrophoresis at pH 6.5. One side strip was stained with bromocresol green (4) to locate any ninhydrin-negative peptides. A second side strip was stained with cadmium/ninhydrin. The remainder of the sample was then eluted off the paper and the peptides collected. In Table II it is seen that the majority of the sample is left with the blocking group still attached (peptide T1-AP1-H1), while a small portion containing the intact peptide is now ninhydrin-positive (T1-AP1-H2). The partial acid hydrolysis conditions released 80% of the phosphate group from T1-AP1. As a control, an unhydrolyzed sample of T1-AP1 showed no phosphorus.

The bond between the serine and asparagyl residue in T1-AP1-H1 is now accessible to penicilliocarboxypeptidase-S and can be cleaved after 2 h (Table IV). These results determined the position of the asparagyl residue as being on the COOH-terminal side of serine 1. This sequence was verified using T1-AP1-H2, a peptide identical with T1-AP1-H1 but devoid of the blocking group (Table II). Although only 6% of the starting material was left with a free NH2 terminus, this amount was sufficient to confirm the sequence at the NH2 terminus of CB3 as being Ser-Asx-Glu-Glu.

Sequence Determination of NH2-terminal Pentapeptide T1-AP2—Residue 5, previously reported as asparagine (10), has been reinvestigated and found to be aspartic acid from the results of penicilliocarboxypeptidase-S treatment of T1-AP2 (Table V). Samples from the digestion were subjected immediately to amino acid analysis at the time intervals indicated. Under the same cleavage conditions for 64 h, standard asparagine was not deamidated. Since the enzyme readily removed aspartic acid after 4 h (unlike the results observed in T1-AP1), it was assumed that serine 1 lacked the phosphate group which somehow prevented the enzyme from cleaving the same bond in T1-AP1. An analysis of T1-AP2 indeed indicated the absence of phosphate.

Identification of NH2-terminal Blocking Group—The rabbit skeletal muscle proteins studied thus far, namely tropomyosin (11), actin (12), myosin (13), troponin-C (14), and troponin-I (15), have all been found to possess an acetyl blocking group at their NH2 termini. When the hydrolyzed samples of the NH2-terminal pentapeptides of tropomyosin, actin, myosin, troponin-C, and troponin-I were subjected to amino acid analyses under the same conditions as used for T1-AP1 and T1-AP2, the results indicated that the blocking group was absent. This is in agreement with the findings of Wellinder and Smillie (7) that this group is not present in the high molecular weight myofibrillar proteins.
Ac-Ser-Asp-Glu-Glu-Val-Val-His-Val-Glu-Glu-Ala-Ala-Pro-Ser-Pro-Ala-Ala-Pro-Ala-Val-His-Glu-Pro-

FIG. 2. Summary of peptides used to establish the complete amino acid sequence of CB3. —, subtractive Edman degradation; ——, penicillocarboxypeptidase-S treatment; ---, regions inferred from amino acid composition and comparison with analogous peptides.

terminal fragment CB3 and a control fragment CB2 were analyzed on the gas-liquid chromatograph, 83% of free acetic acid was recovered from CB3 but none from CB2 (Table VI). It was therefore concluded that Tn-T, like the other myofibrillar components, is an N-acetylated protein.

Peptides from Thermolytic Digestion of T1-t1 - In order to align the peptides T1-AP4 to T1-AP8 obtained from the α-lytic protease digestion of T1 (see Fig. 2), a thermolytic digest was carried out on T1-t1. The four peptides obtained are summarized in Table VII. Peptide T1-t1-Th2 was sequenced through seven residues to overlap T1-AP6 and T1-AP7. T1-t1-Th3 provided an overlap for T1-AP7 and T1-AP8. The two peptides T1-t1-Thii and T1-t1-Thiii had the same mobility at pH 6.5 and were co-eluted off paper. Asparagine and histidine, unique to T1-t1-Thiii, were used to determine its yield as well as the values of other amino acids in its composition. Since the NH₂ terminus of T1-t1-Thii is blocked, the results obtained from the manual sequencing of the two peptides simultaneously were attributed to peptide T1-t1-Thiii. The latter peptide was used to align T1-AP4 and T1-AP5.

Peptides from Peptic Digestion of CB3 - A peptic cleavage of CB3 provided the necessary overlap peptides for the three remaining regions on either side of residues 8-9, 20-21, and 64-65. Table VIII is a summary of the four peptic fragments. P3 stretched through peptides T1-AP10 and T2 (see Fig. 2), confirming the COOH-terminal sequence of CB3. The large peptide P2 consisted of the 47 residues in the central region of CB3. It was taken through 16 manual Edman cycles, thereby
ordering the alytic peptides T1-AP4, T1-AP5, and T1-AP6 as well as the thermolytic peptides T1-ti-Th1ii and T1-ti-Th2. Phenylcillarcoxypeptidase-S was used to confirm the COOH-terminal portion of this peptic fragment (Table IX). The decapeptide P1 had a blocked NH$_2$ terminal; it provided a 2-residue overlap for peptides T1-AP1 and T1-AP4. In order to characterize P1 further, the peptide was subsequently digested with alytic protease. Partial cleavage occurred at valines 5 and 8, resulting in the four peptides shown in Table VIII. Peptides P1-AP1 and P1-AP2 were identical with T1-AP1 and T1-AP2, respectively.

Complete Amino Acid Sequence of CB3 - Fig. 2 summarizes the peptides used to establish the complete sequence of CB3. Aspartic acid 2 was determined from the phenylcillarcoxypeptidase-S results of T1-AP2. Glutamine 65 and asparagine 66 were assigned directly from the mobility of peptide T2-t4 after high voltage paper electrophoresis at pH 6.5. The amide in residue 61 was assigned by determining the mobility after each step of the degradative Edman procedure for peptide T1-t3-P2. The remaining aspartyl and glutamyl residues were found to be acids from mobilities of smaller peptides isolated after high voltage paper electrophoresis at pH 6.5.

The following peptides, commencing from the NH$_2$-terminal end of CB3, provided a minimum overlap of 2 residues: P1, T1-AP4, P2 (T1-t1-Th2, T1-AP7, T1-t1-Th3, T1-AP8, T1-t2, T1-AP9), T1-t3, P3 (and T2). In addition to these overlapping peptides, several others have been included in Fig. 2 to show details of the manual sequencing results.

CB3, consisting of 70 amino acids, is located at the NH$_2$-terminal end of Tn-T. As found in other rabbit skeletal muscle proteins, its NH$_2$ terminus is blocked by an acetyl group. If the phosphate group on serine 1 is assumed to have a charge between −1 and −2 at pH 6.5, then CB3 has a net overall charge of −12 to −13. The occurrence of acidic and basic residues in clusters of 3 is not as prominent as in CB2. The eight prolines are scattered throughout the region of residues 17 to 50.

Amino Acid Sequences of Methionine-containing Peptic Peptides from Tn-T - Fig. 3 shows the isolation of the methionine-containing peptic peptides by Chromobøk P chromatography of the Dowex 1-X2 fractions from the peptic digest of Tn-T. Where necessary, these peptides were further purified by paper electrophoresis and Tables X and XI show the analyses obtained for the purified peptides. Peptide I1-B1 did not yield sequence information, presumably because of modification of its NH$_2$-terminal residue after the peptic digestion. These peptides were used to align the CNBr fragments of Tn-T as shown in Fig. 4.

Amino Acid Sequence of Fragment Obtained by BNPS-skatole Cleavage of Tn-T - Fractionation of the BNPS-skatole digest on Sephadex G-75 gave three peaks (A, B, and C, Fig. 5). Peak A contained uncleaved Tn-T and an M$_r$ = 31,000 fragment, peak B represented a homogeneous M$_r$ = 6,000 fragment, and peak C contained free lysine in addition to other non-amino acid materials which were presumably side products of the cleavage reaction. The partial amino acid sequence of peak B material is shown in Table XII. This sequence was used to align several of the cyanogen bromide fragments of Tn-T as shown in Fig. 4.

Alignment of Cyanogen Bromide Fragments of Tn-T - On the basis of the NH$_2$- and COOH-terminal sequences of the cyanogen bromide fragments CB1 to CB7, and the sequences of the methionine-containing peptic peptides and peak B material from the BNPS-skatole digest of Tn-T, an unambiguous alignment of the cyanogen bromide fragments can be made.

Although the complete sequences of some of the overlap peptides were not determined, partial sequences still established the unambiguity of the order of the fragments. Overlap peptide PII-A3 establishes the order CB3 → CB2 as only fragment CB2 has glutamate as an NH$_2$-terminal residue. Peptic peptide PIA establishes the order CB2 → CB3 as only this combination of cyanogen bromide fragments has glycine, alanine, asparagine, and two serine residues located around a methionine residue. The order CB5 → CB4 is established by the amino acid composition of PII-B1, a peptide which did not sequence presumably because of cyclization of its NH$_2$-terminal residue, glutamate. The order CB4 → CB7 → CB6 is established by the sequence of BNPS-skatole digest Peak B which extends from residues 206 to 258; this alignment is further confirmed by peptides PII-B and PII-C which independently establish the order CB4 → CB7 and CB7 → CB6. These alignments are in agreement with those recently determined independently by Jackson et al. (16).

Amino Acid Sequence of Tn-T - Tn-T consists of 259 amino acids, calculated by sequence analysis to result in a molecular weight of 30,503. This value is substantially less than 37,000, the molecular weight previously estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The anomalously higher values obtained by the latter method have been observed in other muscle proteins, and may be a reflection of the degree of binding of sodium dodecyl sulfate to proteins with a high proportion of charged amino acid residues.

The complete sequence of Tn-T is shown in Fig. 4. A distinctive feature of the sequence is the high proportion of charged residues (approximately 50%) at neutral pH. Thus, of a total of 259 amino acids, there are 61 acidic (aspartyl and glutamyl) and 70 basic (arginyl, lysyl, and histidyl) residues, resulting in an overall charge of +9. These charged moieties, far from being randomly distributed, appear to be arranged in clusters of 3 to 7 residues in length. Furthermore, the NH$_2$-terminal portion (residues 1 to 39) is highly acidic, having 18 aspartates and glutamates and only 4 basic (histidyl) residues. In contrast, the COOH terminal "tail" of Tn-T (residues 221 to 258) is highly positively charged with 13 basic and only 2 acidic residues. The remainder of the molecule has a more regular distribution of acidic and basic amino acids. Recent years studies have shown that Tn-T binds to troponin-C (17-22) and possibly to troponin-I (23). It is conceivable that the acidic NH$_2$-terminal, and basic COOH-terminal extremities of Tn-T represent binding sites for the basic troponin-I (inhibitor protein with isoelectric point of 9.3 (24)) and the highly acidic troponin-C (calcium-binding protein (14), respectively.

Further inspection of the sequence of Tn-T indicates that, apart from one stretch of 14 residues (147 to 160), there are no areas longer than 5 amino acids in length which are devoid of acidic or basic moieties. These observations would indicate that in the tertiary structure of this protein the relative proportion of nonpolar core to surface residues is considerably smaller than in more typical globular proteins.
Sequence of Rabbit Skeletal Troponin-T

<table>
<thead>
<tr>
<th>10</th>
<th>Ac-Ser-Asp-Glu-Glu-Val-Glu-His-Val-Glu-Glu-Ala-Glu-Asp-Val-Pro-Ser-Pro-Ala-Pro-Glu-His-Val</th>
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<tr>
<td>20</td>
<td>Val-Pro-Glu-Glu-Val-His-Val-Glu-Lys-Arg-Leu-Thr-Ala-Pro-Lys-Ile-Pro-Glu-Asp-Val-Asp-Asp-Ile-Val</td>
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<td>30</td>
<td>Lys-Asp-Glu-Glu-Val-His-Glu-Glu-Lys-Arg-Leu-Asp-Ala-Glu-Glu-Glu-Ala-Pro-Ser-Pro-Ala-Glu-Val-His-Glu-Pro-Ala-Pro-Glu-His-Val</td>
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<tr>
<td>40</td>
<td>Val-Ala-Lau-Lys-Cle-Arg-Arg-Ala-Pro-Leu-Glu-Leu-Gln-Ala-Leu-IleAsp-Ser-His-Ph~Glu-Ala-Arg-Lys-Lys-Glu-Glu-Glu-Glu-Leu-</td>
</tr>
<tr>
<td>50</td>
<td>PI-A</td>
</tr>
<tr>
<td>60</td>
<td>Arg-Leu-Ala-Glu-Lys-Ala-Arg-Arg-Val-Asp-Ala-Lys-Asp-Ala-Glu-Asp-Leu-Lys-Lys-Ala-Leu-Ser-Ser-</td>
</tr>
<tr>
<td>70</td>
<td>Met Gly Ala Asn-Tyr-Ser-Set-Tyr-Leu-Ala-Lys-Asp-Gly-Gly-Lys-Gln-Thr-Ala-Arg-Glu-Met-Lys-Lys-Ile-Leu-</td>
</tr>
<tr>
<td>80</td>
<td>CB5</td>
</tr>
<tr>
<td>90</td>
<td>Ala-Glu-Arg-Arg-Pro-Leu-Asn-Ile-Asp-His-Leu-Ser-Asp-Ala-Glu-Leu-Asp-Ala-Arg-Glu-Leu-Trp-Anp-Thr-Leu-Gln-</td>
</tr>
<tr>
<td>100</td>
<td>CB4</td>
</tr>
<tr>
<td>110</td>
<td>Leu-Glu-Thr-Asp-Phe-Glu-Phe-Gly-Glu-Lys-Arg-Glu-Ile-Met-Ala-Arg-Glu-Ala-Glu-Met-Leu-Ala-</td>
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<tr>
<td>120</td>
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</tr>
<tr>
<td>130</td>
<td>Lys-Phe-Ser-Lys-Ala-Gly-Thr-Thr-Ala-Lys-Gly-Val-Gly-Gly-Arg-Trp-Lys</td>
</tr>
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</table>

FIG. 4. The complete amino acid sequence of Tn-T. The cyanogen bromide fragments (CB2 to CB7) are indicated. CB1 is the sum of CB3 and CB2 and results from incomplete cleavage at methionine 70.

The protein could be organized into two or more nonpolar domains linked through regions of structure relatively accessible to interaction with solvent and other protein molecules. Such an arrangement would be consistent with the observed susceptibility of Tn-T to proteolytic degradation during its isolation.

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REFERENCES

Sequence of Rabbit Skeletal Troponin-T

The primary structure of rabbit skeletal muscle troponin-T is described in Table III. The sequence is derived from the cDNA clone 998. The amino acid composition is given in Table IV. The polypeptide fragments are consistent with this composition. The sequence was determined by automated Edman degradation of the tryptic peptides. The results are in excellent agreement with the calculated values. The sequence is consistent with the known function of troponin-T in muscle contraction.
Primary structure of rabbit skeletal muscle troponin-T. Sequence determination of the NH2-terminal fragment CB3 and the complete sequence of troponin-T.

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