Amino Acid Sequence of Rabbit Skeletal Muscle 
$\alpha$-Tropomyosin

THE COOH-TERMINAL HALF (RESIDUES 142 TO 284)*

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Amino acid sequence analysis of the large cyanogen bromide fragment (residues 142 to 281) derived from the COOH-terminal half of the mixed tropomyosin population of rabbit skeletal muscle has been carried out. The isolation and sequence analysis of peptides derived from chymotryptic and cysteic protease digest have confirmed certain overlaps. Based on previously published data the sequence can be extended to residue 284, the COOH-terminal end of the protein. In fourteen positions, amino acid substitutions have been observed. In one of these (residue 199) the sequence evidence indicates a minimum of four different polypeptide chains in the mixed tropomyosin population. The assignment of particular amino acid residues to these positions for the major $\alpha$-component of rabbit skeletal tropomyosin has been based on the relative recoveries of peptides containing different residues in these positions.

Tropomyosin, associated with the troponin complex, is known to be intimately involved in the calcium regulatory system of skeletal muscle. Located in each of the two grooves of the two-stranded actin helix it consists of a polar filament composed of a head-to-tail assembly of individual molecules, each of which is in contact with 7 (or 14) actin monomers. To each tropomyosin molecule is bound one complex of troponin made up of three components: the calcium-binding protein (troponin-C), the inhibitory protein (troponin-I), and the tropomyosin-binding protein (troponin-T). As a result of a conformational change induced in the troponin complex by the binding of calcium to troponin-C, tropomyosin alters its position in the groove of the actin filament permitting the interaction of myosin heads and actin monomers (for review see Weber and Murray (1)).

Tropomyosin may be considered as the smallest and simplest of the $\alpha$-group of fibrous proteins, which also includes $\alpha$-keratin, paramyosin, and the rod region of myosin. Because of its ready solubility in aqueous solutions its physical and chemical properties have been extensively studied (2-7). In concentrated salt solutions its molecular weight is about 66,000, a value reduced to 33,000 in the presence of denaturants under reducing conditions. This dissociation into two polypeptide chains is accompanied by a reduction in its helical content from a value close to 100% to zero as estimated by its optical rotatory dispersion and circular dichroism properties. In dilute salt solutions the rodlike protein molecules aggregate end to end to form long filaments (8, 9). Under appropriate ionic conditions the protein forms true crystals which, however, are 95% water and show considerable disorder. A variety of polymorphic forms have been observed (10-16), all of which appear to result from cross-connections between long molecular filaments. Estimates of the effective molecular length in these crystals provide a value of 410 ± 4 Å (11). Under other conditions in the presence of divalent cations, the protein forms highly ordered fibrous aggregates, tactoids, or paracrystals, which in the electron microscope after staining with uranyl salts show a detailed banding pattern with a period of 395 Å (10, 13, 17, 18).

Crick (19) proposed that the main features of the x-ray diffraction pattern of the $\alpha$-group of fibrous proteins were explicable in terms of a coiled-coil structure which, in the case of tropomyosin, would consist of two right-handed helices wrapped around one another to form a two-stranded rope. If the two helices are inclined one to the other by about 20°, the side chains of one helix will interact with the side chains of the other in a knobs-into-holes manner. In order for the $\alpha$-helices to remain in contact indefinitely, it is necessary to deform them slightly along their entire length. As pointed out by Crick (19) it was plausible to postulate that a structure of this type would be stabilized by the occurrence of nonpolar residues between the chains while the polar and ionic residues would be situated on the outside of the molecule where they would interact with the solvent. This predicts a repeating pattern of nonpolar and polar residues in the amino acid sequence of such a structure, with the nonpolar occurring at an average interval of 3.5 residues so that they always

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point toward the inside of the molecule. To test these penetrating analyses we undertook the elucidation of the amino acid sequence of rabbit skeletal tropomyosin. In addition to providing information on the relationships between amino acid sequences and coiled-coil structures, it was anticipated that a knowledge of the primary structure of tropomyosin would lead to a fuller understanding of its role in the relaxing protein system; i.e., the structural basis of its interaction with actin and the troponin system as well as its assembly into long filaments. Such information was also anticipated to be of value in the interpretation of the staining patterns of paracrystals of the protein as well as providing essential information for the eventual description of its tertiary structure from x-ray diffraction studies.

Initial sequence studies were directed toward the question of the identity or nonidentity of the polypeptide chains of the protein. Rabbit skeletal muscle was chosen as the tissue source since the bulk of the available chemical and physical evidence had been accumulated with this tropomyosin. Determination of the numbers of unique sequences about the cysteine residues was inconsistent with a homogeneous preparation (20), a result in agreement with indications of heterogeneity from polyacrylamide gel electrophoresis (6, 21). However, the numbers of unique histidine and methionine sequences (23) indicated that the constituent polypeptide chains were highly similar in amino acid sequence. Since several attempts to separate these chains in our laboratory were unsuccessful we were encouraged to undertake the sequence analysis on the unfractionated material. After cyanogen bromide cleavage, the fragments could be separated on Sephadex G-50 or G-75 into two fractions, one of high molecular weight, Cn1 (M, = approximately 30,000) and the other, Cn2, containing a number of small peptides. The fractionation and sequence determination of the small peptides has been described previously (23). The high molecular weight fraction was further separated into two components, one making up the bulk of the COOH-terminal half of the molecule (residues 142 to 293), designated Cn1, and the other, the N-terminal half (residues 11 to 127), designated Cn1A. The present paper documents the sequence determination of fragment Cn1B derived from the mixed tropomyosin population. The subsequent paper (24) describes the elucidation of the sequence of the Cn1A fragment which has been prepared from α-tropomyosin, subsequent to the description by Cummins and Perry (25) of a chromatographic procedure for the separation of the two major tropomyosin components, α and β. This information, coupled with the previously determined methionine sequences (22), has permitted the assignment of a complete amino acid sequence for α-tropomyosin. Some of the results of these studies have previously been published elsewhere (26-28).

**MATERIALS AND METHODS**

Tropomyosin from rabbit skeletal muscle was prepared as previously described (22). The preparation of 14C-labeled S-carboxymethylated tropomyosin (Cn-tropomyosin), the cyanogen bromide cleavage reaction, and the isolation of the cyanogen bromide fragment Cn1B were carried out as before (23). The enzymes α-chymotrypsin (three times crystallized) and 1-lysozyme (2-phenolyl chloromethyl ketone-lysozyme) were purchased from Worthington. Thermolysin (crystalline, B grade) was obtained from Calbiochem. The α-lytic protease of Myxobacter 495 was prepared by Dr. D. R. Whittaker's laboratory, University of Ottawa (29). Technicon Chromobeads type P (peptides), equivalent to Dowex 50-X4, were obtained from Technicon Chemical Co., Inc., Chauncey, N. Y., and A. J. P. A. Dowex 1-X2 resins were from Bio-Rad. Sources of G-25 (fine), G-50 (fine), and G-75 (superfine) were bought from Pharmacia. Aqueous scintillation fluid was purchased from New England Nuclear. N-Ethylmorpholine (practical grade) was obtained from Eastman, and redistilled (in 150°C) before use. The inhibitor, diisopropyl fluorophosphate, was from Schwarz/Mann. All other chemicals were reagent grade and used without further purification.

**Maleylation and Denmaleylation** — The procedure was based on that described by Butler et al. (30). The Cn1B fragment (7 μmol) was dissolved in 1 ml of 0.1 M phosphate buffer, pH 7.0. A 20-fold molar excess of maleic anhydride (220 mg) over total lysines was dissolved in a minimal quantity of dioxane (0.5 ml) and added in small portions with rapid stirring over 1 h at 25°C. The pH was maintained at 9.0 by automatic titration in a pH-stat with 1 N NaOH. The product was then exhaustively dialyzed against 0.5% ammonium bicarbonate and lyophilized. For denmaleylation of side chain hydroxy groups, the method of Freedman et al. (31) was followed. The maleylated fragment was dissolved in 0.8 M hydroxylamine, pH 6.5, and incubated at 4°C for 16 h. After dialysis against deionized water the product was lyophilized. For total denmaleylation, peptides were adjusted to pH 3.0 to 3.5 by the addition of glacial acetic acid and incubated at 60°C for 8 h.

**Proteolytic Digestions** — A limited chymotryptic digestion of Cn1B (172 mg) was carried out in a pH-stat at 25°C in 0.1 M KCl at a concentration of 10 mg/ml of 0.1 M phosphate buffer, pH 8.0. With thermolysin the buffer contained 0.05 M CaCl. Unless otherwise specified the enzyme to substrate molar ratio was 1:100. After the initial rapid phase of the digestion (4 h) the reaction was terminated by adding diisopropyl fluorophosphate to a concentration of 10 mM and lyophilization. Measurement of the amount of alkaline consumed indicated that 14 to 15 peptide bonds had been cleaved assuming an average pK of 7.65 for α-amino groups.

Other proteolytic digestions of the unmodified or maleylated fragment, or of smaller peptides by trypsin and α-lytic protease were carried out in 0.05 M N-ethy1morpholinelacetate buffer at pH 8.0. With thermolysin the buffer contained 0.05 M CaCl2, and otherwise specified the enzyme to substrate molar ratio was 1:100. Chymotryptic, tryptic, and α-lytic protease digests were terminated by the addition of diisopropyl fluorophosphate to a concentration of 10 mM and thermolytic digestions by freezing and drying.

**Isolation and Purification of Peptides —** Sephadex columns of G-25, G-50, and G-75 were prepared by swelling the beads in 0.05 M N-ethylmorpholinelacetate buffer, pH 8.0, followed by several deaerations to remove fines. Before packing the columns, the slurries and developing buffer were deaerated. Each column was equilibrated with the above buffer for at least 24 h before applying the sample. Column effluents were monitored for absorbance at 230 and 280 nm. Where appropriate, fractions were monitored for radioactivity by mixing 25-μl samples with 10 ml of Aquasol and counting in a Packard liquid scintillation counter.

**Chromatography of peptides on Chromobeads type P** resin and on AG 1-X2 (Dowex 1) resin was carried out as previously described (22, 23). A preliminary batch fractionation on AG 1-X2 resin of the complex mixture of peptides arising from α-lytic protease digestion of fragment Cn1B was carried out by the method described by Johnson and Smillie (33). High voltage electrophoresis of peptide mixtures, amino acid analysis, manual sequence analysis by the dansyl-monitored Edman method, and assignment of amines from electrophoretic mobilities have all been previously described in detail (20, 22, 23).

**Nomenclature of Peptides** — Peptides have been designated according to the enzyme used for cleavage as follows: T, trypsin; C, chymotrypsin; Th, thermolysin; 1, α-lytic protease. Peptide fractions arising from tryptic digestion of maleylated fragments have been designated TM.

**RESULTS AND DISCUSSION**

**Strategy for Sequence Determination of Fragment Cn1B** — Preliminary studies of this fragment indicated that the NH2-terminus was at least partially blocked, probably as the methyl ester. Some of the data are presented as a miniprint supplement, immediately following this paper. Figs. 1 to 3 and 5 to 7 and Tables I to VIII are found on pp. 1134-1136. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document Set 77M-1327, cite author(s), and include a check or money order for $4.80 per set of photocopies.
Sequence of Rabbit Skeletal Tropomyosin

Fig. 4. Summary of sequences elucidated from characterization of chymotryptic peptides (see Tables I, II, and III) coupled with sequences previously determined (20, 22) from trypsin, thermolytic, and peptic digests of tropomyosin. The latter peptide sequences have been designated with an asterisk. For clarity, residue numbers correspond to those in the final sequence of α-tropomyosin (24, 28).
result of cyclization to pyrrolidone carboxylate. This ruled out the use of automated or manual Edman degradation of the whole fragment. Since it was possible that tropomyosin might contain a regular repeat of similar polypeptide sequences and since we knew that the fragment was heterogeneous because of the presence of several similar polypeptide chains, we looked for a procedure of fragmentation that would provide a small number of fairly large peptides. In this way any ambiguities due to the heterogeneity would be minimized. An examination of the amino acid analysis of Cn1B indicated a low number of aromatic amino acids; 5 tyrosines and 1 phenylalanine//chain. For these reasons a limited chymotryptic digestion was carried out and the resulting peptides isolated and characterized. In addition, a trypic digest of the maleylated fragment cleaved the polypeptide chain at its 6 arginines. Sequence analyses of these peptides, combined with the results of the chymotryptic peptides, allowed the formulation of a complete sequence for Cn1B. To consolidate the overlaps a third digestion of Cn1B with α-lytic protease of Myxobacter 495 was done. Isolation and characterization of the resulting complex mixture of peptides confirmed the overall sequence.

Chymotryptic Peptides— This digest was fractionated by gel filtration on Sephadex G-25 into Fractions CI to CV (Fig. 1). Peptides in Fractions CII to CIV were further purified by high voltage electrophoresis. Peptides of Fraction CI were separated by gel filtration on Sephadex G-50 (Fig. 2), ion exchange chromatography on AG 1-X2 (Fig. 3), and final purification by chromatography on Chromobeads type P and/or by high voltage electrophoresis. The properties, amino acid compositions, and sequence information of each of the peptides are documented in Tables I, II, and III.

A summary of the evidence for the amino acid sequences elucidated from the peptides derived from the limited chymotryptic digestion is assembled in Fig. 4. Included in this summation are sequences previously elucidated (20, 22) about the 2 histidines (residues 153 and 276) and a cysteine (residue

Fig. 8. Summary of evidence for sequence of residues 142 to 284 of tropomyosin. Sequences I to XI, derived from chymotryptic digests, are taken from Fig. 4. Evidence for peptides derived from tryptic digest of maleylated Cn1B (TM peptides) has been summarized in Tables IV, V, VI, and VII. Selected peptides (L peptides) from α-lytic protease digestion of Cn1B have been documented in Table VIII. In residue positions where amino acid substitutions occur, the upper amino acid is found in peptides in highest yield. Only at residue 189 has more than one substitution been detected.
190) as well as an overlap for cyanogen bromide fragments Cn1B and Cn2-III at the COOH-terminal end of the tropomyosin molecule(s). These previous sequences have been fully confirmed by the present work.

From the detailed sequence information presented in Tables I, II, and III it was possible to assemble 11 sequences numbered I to XI, shown in Fig. 4, which account for over 90% of the total sequence of this cyanogen bromide fragment. In spite of the fact that the sequence analysis was complicated by the presence of a number of positions in which amino acid substitutions were present (13 detected), the similarity between the sequences of the isolated peptides was sufficient to assign them with confidence. In one position (residue 199 of sequence III), more than one residue substitution occurs. Thus, threonine (occurring in peptides recovered in greatest yield) can be replaced by isoleucine or lysine. Both threonine and lysine have been found to occur in peptides in which residues 191 and 192 are alanine and glutamate, respectively, while isoleucine and lysine have been observed in peptides in which residues 191 and 192 are glycine and aspartate, respectively. This indicates that the mixed tropomyosin population is made up of at least four different polypeptide chains whose sequences are determined by multiple genes.

Tryptic Peptides of Maleylated Cn1B—The peptides (TM peptides) derived from a tryptic digest of maleylated Cn1B were fractionated by a combination of gel filtration on Sephadex G-75 (Fig. 5), ion exchange chromatography on Chromobeads type P (Fig. 6), and by high voltage electrophoresis at pH 6.5, 3.5, and/or 1.8. The amino acid compositions of these peptides are given in Table IV and their properties and sequence analyses in Tables V, VI, and VII. These data coupled with the sequences I to XI derived from the chymotryptic peptides permitted the assignment of the complete sequence for the Cn1B fragment as summarized in Fig. 8.

Confirmatory Overlap Peptides from α-Lytic Protease Digestion of Cn1B—Although the sequence derived from the chymotryptic peptides and the tryptic peptides from maleylated Cn1B was consistent with the amino acid composition of Cn1B and with all available data, the overlap evidence in the region of residues 205 to 206, 213 to 214, and 264 to 268 was weak. For confirmation of the complete sequence in these regions a digestion of Cn1B with the α-lytic protease of Myxobacter 495 was carried out. Since this enzyme has a unique specificity for peptide bonds involving amino acids with uncharged side chains and does not cleave at basic or aromatic residues, it was expected that suitable overlaps would be obtained. The complex mixture of peptides resulting from this digestion was fractionated by gel filtration on Sephadex G-25 and G-50, batch fractionation on AG 1-2X, ion exchange chromatography on Chromobeads type P, and high voltage electrophoresis at pH 6.5, 3.5, and/or 1.8. By these methods some 60 peptides were recovered in adequate amounts and purity for characterization and sequence analysis. Of these, we report here (Table VIII) only those pertinent to the establishment of the overlaps indicated above. In addition, one peptide (LII-q2) indicated the presence of a methionine substitution for leucine at residue 265 which had not previously been detected. The properties and sequence information of these peptides are summarized in Table VIII and their positions in the complete sequence of Cn1B are shown in Fig. 8.

As a result of these analyses it has been possible to assign with confidence a complete sequence for residues 143 to 284 of tropomyosin making up almost exactly the COOH-terminal half of the molecule. All amides and acids have been assigned with the exception of residues 142, 144, and 145. These assignments are made in the subsequent paper (24). Since the preparation of tropomyosin used in these studies was a mixture of two components, α and β, present in a molar ratio of 3 to 1 (25), 14 residue positions have been observed where amino acid substitutions occur. Although this heterogeneity has complicated the sequence analysis, the substitutions have been conservative in nature and the isolated peptides have been sufficiently similar that their positions in the sequence could be assigned with confidence. The assignment of particular amino acids for the major α-component of tropomyosin to those residue positions where substitutions have been observed has been based on the relative recoveries of peptides containing different amino acids in these positions. The assumptions implicit in these assignments may not in all cases be valid and may eventually require some minor modifications in the sequence of the COOH-terminal half of α-tropomyosin as it is reported here. However all indications to date from sequence studies currently underway on the purified α- and β-components would indicate that these assignments have been correct. In any case, the occasional misassignment of one similar amino acid for another is not likely to materially affect the interpretative studies of the sequence recently published by several authors (35-38).

REFERENCES

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Sequence of Rabbit Skeletal Tropomyosin
Amino acid sequence of rabbit skeletal muscle alpha-tropomyosin. The COOH-terminal half (residues 142 to 284).
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