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Tropomyosin and its Tryptic Fragments: Non-Identical Chains and Submolecular Structures

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Tryptic fragments of tropomyosin collected by isoelectric precipitation at pH 4.6 showed three bands on SDS gel electrophoresis, two major bands corresponding to apparent molecular weights of 16,000 and 12,000, and minor one to 28,000. Those components were separated on a QAE sephadex column at pH 8.6 by increasing KCl concentration from 0.24 M to 0.5 M. For the purpose to identify the components, amino acid residues in the neighborhood of *C*-terminals of intact tropomyosin and the components were determined by the treatment of carboxypeptidase A.

1) *C*-terminals of intact tropomyosin are deduced to be isoleucine and leucine, suggesting that the two chains which constitute a coiled-coil structure are different.

2) The first component has a sequence to the *C*-terminal of -Met-Leu-Lys, and the last two components -Thr-Ser-Ile, which is one of the intact chains.

These results are discussed in connection with a submolecular structure of tropomyosin.

INTRODUCTION

Tropomyosin, which was isolated and characterized first by Bailey,[1] is a typical fibrous protein extracted from skeletal muscle. Similar to paramyosin and light-meromyosin, tropomyosin is an almost fully α-helical[2] and rod-shaped molecule with the length of about 400 Å and the width of about 20 Å, whose polypeptide chain conformation is expected to be coiled-coil.[3] The molecular weight obtained up to the present time lies from about 60,000 to 70,000.[4~7] Equilibrium sedimentation measurements in the presence of 8 M urea with and without 0.1 M β-mercaptoethanol, however, showed a molecular weight of 34,000,[7,8] and more recently Weber and Osborn reported 37,000 also by SDS gel electrophoresis.[9] Partial sequence of one chain of tropomyosin[10] demonstrates characteristic pattern of repeated appearance of hydrophobic amino acid residues along the chain, which could be energetically favorable for the formation of a coiled-coil structure. All of these results suggest that tropomyosin consists of the same or different but similar two polypeptide chains. Since we have no detailed works on complete *C*-[11~13] and *N*-terminals[14~17] of the protein, it will be worthwhile to examine the submolecular structure of tropomyosin and the stability of the structure more in detail.

MATERIALS AND METHODS

Preparation of Tropomyosin

Tropomyosin was prepared from rabbit skeletal muscle according to the proce-

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dure of Bailey in principle.[1] Crude tropomyosin was extracted from dry muscle or native tropomyosin. Isoelectric precipitation in the presence of 1 M KCl and the subsequent salting out from 47 to 55% ammonium sulfate saturation were repeated three to four times for the purification. The purity was checked by the ultraviolet absorption spectra after removing ammonium sulfate by dialysis against water. Since tropomyosin contains neither tryptophan residue nor nucleotide, the absorption spectrum should be that for tyrosine with maximum at 277 nm and minimum at 252 nm. Protein concentrations were determined by the optical density at 277 nm (0.25/mg/ml for tropomyosin and 0.27/mg/ml for tryptic fragments:[18] these values are consistent with the estimated value from the number of tyrosine and the molar extinction coefficient of tyrosine at 277 nm, 1500).

Tryptic Digestion of Tropomyosin

Following three methods were empolyed in obtaining tryptic fragments;

a) Tryptic digestion of tropomyosin was carried out at room temperature with an automatic pH-stat (Radiometer TTTl). Detailed procedure was described elsewhere.[18]

b) A tropomyosin solution of a concentration of 5 to 6 mg/ml was incubated with trypsin (a weight ratio to the protein=1: 300) at 0°C in the presence of 0.05 M KCl and 0.01 M tris-HCl buffer of pH 7.8. The digestion was terminated by the addition of acetate buffer of pH 4.6 to the reacted solution.

c) Digestion in methanol-water system was performed in 10% (v/v) solution at room temperature, and the procedure used in as a) and b) was employed to separate fragments.

Carboxypeptidase Digestion

A solution of tropomyosin or tryptic fragments containing 0.1 M KCl and 0.01 M tris-HCl buffer of pH 8.0 was incubated with carboxypeptidase A (DFP treated, Sigma Chemicals) at 41°C. At this temperature tropomyosin molecules are dispersed in depolymerized form. After the addition of the enzyme (a weight ratio to the protein=1:400) to a solution, an aliquot was removed from the reacted mixture at appropriate time intervals, and was mixed immediately with trichloroacetic acid to stop the reaction and the precipitated protein was spun down. Amino acids liberated in the supernatants were analyzed on an automatic amino acid analyzer (Jeol Model JLC B3).

Gel Electrophoresis

The procedure adopted for SDS polyacrylamide gel electrophoresis was essentially the same as the method employed by Weber and Osborn.[9] Before electrophoresis the proteins in 0.01 M sodium phosphate buffer of pH 7.0, 0.1% SDS and 1% β-mercaptoethanol were incubated at room temperature. Electrophoresis was performed at a constant current of 8 mA per gel for about 4 hours. After the electrophoresis the gels were stained for 1 h. amino black 10B containing 7% acetic acid and destained by multiple changes of 7% acetic acid for 24 hours or by electrodialysis.

Apparent molecular weights were determined by migration distances with several marker proteins of well-characterized molecular weights.

Fractionation of Tryptic Fragments

Column chromatography of tryptic fragments on QAE sephadex A50 (1.5×20 cm) was carried out at room temperature, equilibrated with 0.02 M tris HCl buffer of pH 8.6, and stepwise or gradient elution with increasing KCl concentration from 0.24 to 0.5 M was used. Fractions were monitored at 280 nm by a colorimeter-recorder system of an automatic analyzer (Jeol Model JLC B3) coupled to a fraction collector. In experiments of preparative scale, effluents were collected into tubes and the absorbance at 277 nm was measured on a Hitachi 200 spectrophotometer.

N-terminal determination was performed by thin layer chromatography after dinitrophenylation of *N*-terminal amino acids.

RESULTS

When gel electrophoresis was carried out immediately after mixing tropomyosin with 0.01 M sodium phosphate buffer of pH 7.0 containing 0.1% SDS and 1% β-mercaptoethanol, two distinct bands appeared; one band corresponded to a molecular weight of about 80,000, and the other band to a molecular weight of 38,000 as found already by Weber and Osborn.[9] The value of 80,000, however, is not reliable since error becomes greater at such a high molecular weight in this gel condition. On the other hand, only one band of 38,000 was observed after incubation of the solution with SDS and β-mercaptoethanol for a few hours.

After a short time incubation with the SDS solvent *without* β-mercaptoethanol, only one band of molecular weight of 80,000 was observed, but after several hours incubation in the solvent, both of two bands of molecular weight of 80,000 and 38,000 appeared. Much longer incubation over 24 hours resulted in complete disappearance of the band of 80,000, indicating the addition of β-mercaptoethanol in the SDS solvent only made the dissociation of tropomyosin into subunits faster, and disulfide bridge, if present, is not required for the maintenance of the native conformation.

A tropomyosin molecule, when digested with trypsin, is fragmented into heavy fragments which are precipitated at pH 4.6, and small acid soluble fragments. A mixture of undigested tropomyosin and the tryptic fragments was collected by isoelectric precipitation at appropriate time intervals. The time course of tryptic digestion at 0°C was followed by SDS gel electrophoresis as shown in Fig. 1. Soon after a short time digestion, three bands appeared, each corresponding to a molecular weight of 38,000, 16,000, and 12,000, respectively. The band of 38,000 corresponds to the undigested tropomyosin subunit, and the two bands of smaller molecular weights appeared simultaneously. As the digestion proceeded further, the band of undigested material became thinner, while both of the two other bands became denser. Even after 70 hours when the bands of tropomyosin had disappeared completely, the two bands remained stable.

The fragments prepared by the digestion at room temperature yielded the same

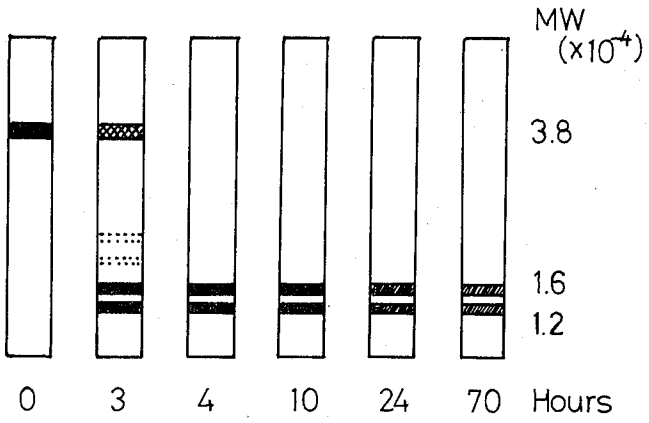

Fig. 1. Schematic representation of acrylamide gel electrophoresis to show the time course of tryptic digestion of tropomyosin at 0°C.

molecular weights of 16,000 and 12,000 on SDS gel electrophoresis as the digest at 0°C. Digested material in 10% methanol medium also showed similar fragments; for short time (15 min.) digestion, a weaker band corresponding to a molecular weight of 28,000 in addition to 16,000 and 12,000 was observed, while this band disappeared after long time digestion (60 min.). The two major bands observed in all the preparations exhibited similar density during the time course of digestion. Therefore, these components are major products which precipitate at pH 4.6 in the presence of 0.05 M KCl.

Molecular weight determination by sedimentation of Archibald technique gave rise to a value of 35,000 in 0.1 M KCl for the fragments obtained at 0°C digestion. This value is consistent with the molecular weight of the fragments prepared with the digestion at room temperature.[18] Since the sum of apparent molecular weights of the components detected on SDS gel electrophoresis is smaller than the molecular weight obtained by the sedimentation experiment, association of the components is expected to occur in the absence of dissociating reagents, as has been observed on CNBr fragments.[17]

Separation of two (digestion at 0°C and room temperature) and three (digestion in 10% methanol) components could be achieved on QAE-sephadex column, although it was impossible to separate the intact two chains of tropomyosin by the same column at various elution conditions presumably due to very similar net charge of the two subunits as suggested by Woods.[7] Figure 2 illustrates the separation of three components, where the third component could not be resolved enough to form a peak. Rechromatography, however, gave rise to good fractions to be used for *C*-terminal determination (Fig. 3). SDS gel electrophoresis on each fraction indicated that component I corresponds to the fragment of molecular weight of 16,000, component II to that of 12,000, and component III to that of 28,000.

A time course of amino acid liberation for intact tropomyosin by the treatment of carboxypeptidase A is shown in Fig. 4. Isoleucine is one of the *C*-terminals of tropomyosin as has been identified by Hodge and Smillie,[13] since it was most rapidly

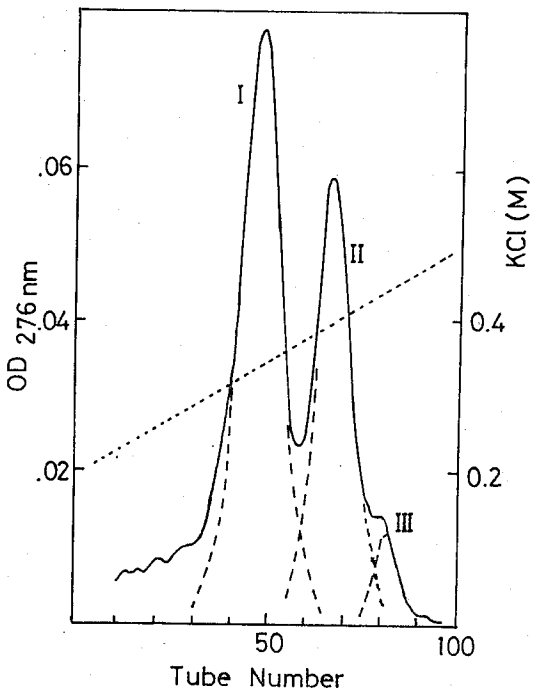

Fig. 2. Chromatogram of tryptic fragments on QAE sephadex column. A linear gradient from 0.2 M KCl to 0.5 M KCl was employed at pH 8.6. Dashed lines represent each component, I, II, and III.

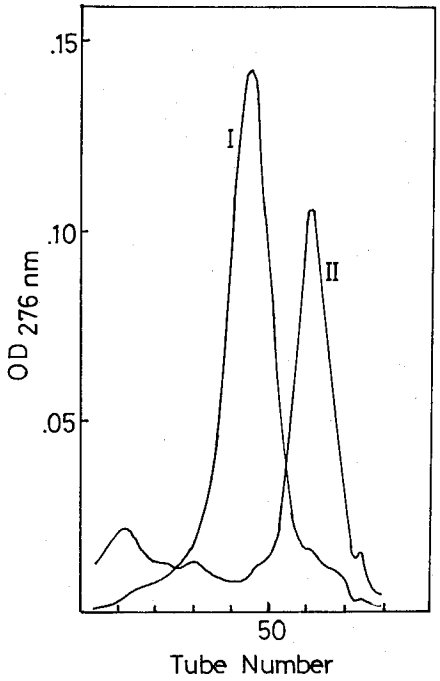

Fig. 3. Rechromatogram of component I and II on the same QAE sephadex column, demonstrating separate runs on the same figure.

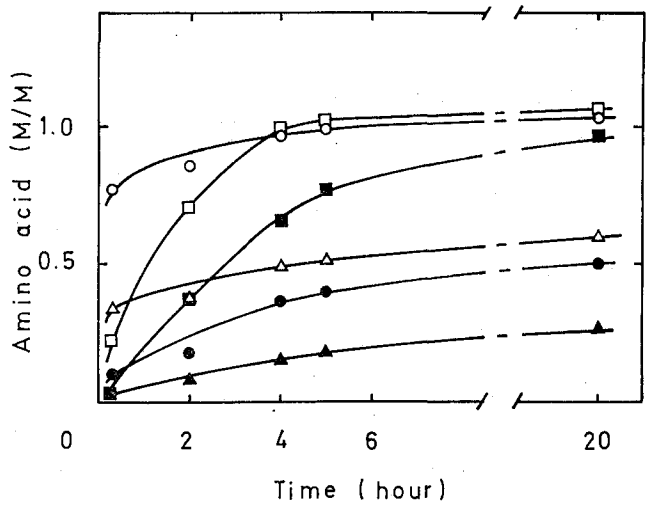

Fig. 4. A time course of amino acid liberation from intact tropomyosin by the treatment of carboxypeptidase A. Unit of the ordinate is mole of amino acid per 60,000 dalton.
○ Isoleucine; □ Serine; △ Leucine; ● Methionine, ■ Threonine; ▲ Aspartic Acid.

liberated and reached the plateau of 1 mol/mol of tropomyosin. Leucine is secondly rapid in liberation at the initial stage. However, the amount of serine and threonine became larger than that of leucine soon. Comparing the time course of liberation of major amino acids, Ile, Ser, Thr, Met, Asp, and Leu, with the partial sequence near *C*-terminal of one chain, -Asp-Met-Thr-Ser-Ile, we may infer that leucine is most likely to be *C*-terminal of another chain. Since no basic amino acid was detected even by the treatment with carboxypeptidase B, subunits of tropomyosin must be non-identical; one has isoleucine, and the other leucine as *C*-terminal. The profile of amino acids in the *C*-terminal portion was always the same for more than fifty preparations as far as the same purification procedure was employed, except a few cases which gave greater Leu in amount than Ile.

Treatment of the unfractionated tryptic fragments with carboxypeptidase A gave the major amino acids liberated as Ile, Leu, Ser, and Thr, just the same as those from intact tropomyosin. Figure 5 demonstrates the time course of digestion, which is essentially similar to that of Fig. 4. However, the liberation of basic amino acids, major lysine and minor arginine, was detected, indicating the presence of some fragment having new *C*-terminal. Nevertheless, the present result suggest that the original *C*-terminal portion would remain in the tryptic fragments.

C-terminal determination on components in tryptic fragments separated by the QAE sephadex column was performed as listed in Table I. From these results, it is deduced that the sequence near *C*-terminal or component I is -Met-Leu-Lys, and the *C*-terminals of component II and component III are Ile. Although an appreciable amount of Leu are present in component II and III, the low yield of a basic amino acid, Lys, indicates that these components have one of the intact *C*-terminals, *i.e.*, -Asp-Met-Thr-Ser-Ile. The *C*-terminal sequence of component I corresponds

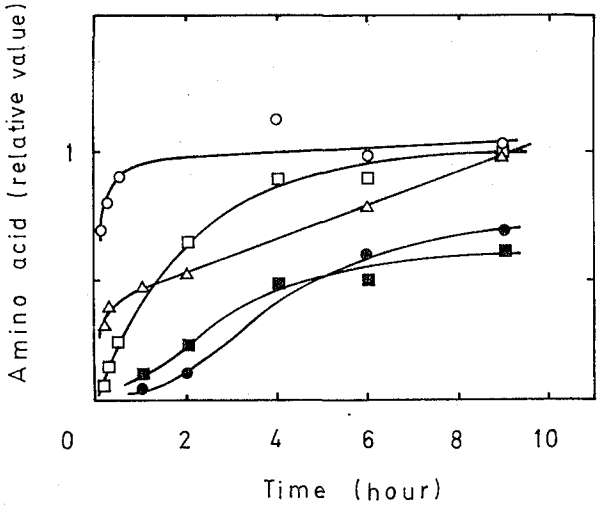

Fig. 5. A time course of amino acid liberation from tryptic fragments of tropomyosin by the treatment of carboxypeptidase A.
○ Isoleucine, □ Serine; △ Leucine; ● Methionine, ■ Threonine.

Table I. Amino acids liberated from component I, II, and III by carboxypeptidase A. (Values are normalized as Lysine for component I and Isoleucine for component II and III, =1.00)

	component I		component II		component III	
	5 min	90 min	90 min	240 min	10 min	120 min
Lys	0.69	1.00	0.16	0.20	—	0.04
Asp	—	—	—	0.50	—	—
Thr	—	—	—	0.70	—	0.07
Ser	—	—	—	0.90	—	0.24
Ala	—	—	—	0.15	—	—
Met	—	0.34	—	1.05	—	—
Ile	—	—	0.49	1.00	0.35	1.00
Leu	0.09	0.60	0.08	0.60	0.08	0.32

to one of the methionine containing peptide reported by Hodges and Smillie.[19] The result on component I is somewhat ambiguous, since most of the preparations of component I yielded Leu and Lys as major amino acids by the treatment of carboxypeptidase but in a few cases a low amount of lysine was obtained. It is possible that component I might be a mixture of polypeptide chains of a similar size which have Leu in the neighborhood of *C*-terminal.

Attempts to determine *N*-terminals of intact tropomyosin did not succeed because of acetylation of amino groups of the terminal residue. Exposure of the molecule, however, at alkaline pH beyond pH 12.0 resulted in appearance of Gly, Ala, and Ser as *N*-terminals, so that quantitative measurements of the residues were performed as a function of time. The amounts of DNP amino acids separated on thin layer chromatography increased linearly with the incubation time in 0.01 N NaOH at

40°C over 1 hour, and the rate of increment was larger at higher temperature and higher the pH (0.1 N NaOH), the final amount being several times greater than moles of subunits. This result indicates that some peptide bonds of tropomyosin are cleaved easily by the exposure at high pH. Gel electrophoresis showed the process of degradation of the molecules with the incubation time, that is, amount of many fast moving bands corresponding to small fragments increased, and finally, original material disappeared completely. Since most of proteins are stable at such a condition, this phenomenon seems to be unusual. The irreversible denaturation of tropomyosin once exposed at a high pH beyond 12 would be due to the cleavage of peptide bonds.

DISCUSSION

The present results show that the *C*-terminals of tropomyosin are isoleucine and leucine, indicating that tropomyosin contains two different chains. Recent success in the separation of tropomyosin into two or four components on CM cellulose column in the presence of urea at pH 4.0[20] would be compatible with the above results. We, however, have no established evidence whether the same kind of polypeptides or two different chains make up a single coiled-coil molecule. If the former is the case, *C*-terminal of one fraction will give a single amino acid, *e.g.*, isoleucine.

Among a number of runs on *C*-terminal determination of tropomyosin prepared by the procedure described in Method, sometimes appreciable amounts of Gly, Ala, and Val were detected in addition to six major amino acids, Ile, Leu, Ser, Thr, Met, and Asp. Since Gly and Val are not located in the neighborhood of *C*-terminal of one chain,[17] these amino acids come from some other chain(s): the leu chain or some protein tightly bound to tropomyosin, *e.g.*, troponin T, or another unidentified protein similar to tropomysoin,. Works to separate the chains are in progress.

It seems to be peculiar that the liberation of amino acids from the Ile chain by carboxypeptidase treatment proceeds faster than other chain(s) (Fig. 4). Even after 10 hours more, we could not confirm any other excess amino acid than Leu with reliable reproducibility. This would be explained in the following ways: on assuming that the two chains stagger by some residues to form a coiled-coil structure[21] as proposed by Hodge *et al*,[17] the Ile chain is more accessible to enzymatic attack than the Leu chain: another possibility is that an amino acid preceding to Leu might not be cleaved by carboxypeptidase A, *e.g.*, proline which can not be released by the enzyme. Separation of two different chain would be useful for the decision of this point.

The analysis of *C*-terminals of components separated on QAE sephadex column shows that component II and III are fragmented from the Ile chain, while it not clear whether component I comes from the Ile chain or from the other chain. If the original chain of component I is the Ile chain, the component must be either *N*-terminal portion of the chain or a part of component III, because those three components appeared simultaneously during the course of digestion. The first pos-

sibility is not likely, since the sum of molecular weights of component I and III, 44,000, exceeds the original value 38,000. The second explanation, therefore, seems to be feasible: the sum of two component, I and II, gives rise to a size of component III. On inspection of the partial sequence of the Ile chain, however, there is no sequence corresponded to Met-Leu-Lys. Thus, it is deduced that component I belongs to the other chain, Leu chain.

Since α-helical conformation would be resistant to tryptic attack, primary positions to be cleaved would have a loose structure. Only from the present results, it is not possible to locate the positions. Preliminary observation of tryptic fragments by an electron microscope showed the existence of shorter rod-like molecule (less than 200 Å) than the intact 400 Å molecule. Since component I and II are resistant to tryptic digestion, these two components are presumed to constitute the fragments observed in the electron microscope.

For the purpose to separate subunits of tropomyosin, we prepared various affinity column which are capable to make linkage to sulfhydryl groups, *e.g.*, by introduction of iodoacetate derivatives in sepharose matrix. In denaturing medium such as 4 M guanidine HCl, tropomyosin did react with the sepharose, but identification of the subunits has not been completed yet, because of poor recovery of the reacted materials, and of peculiar reaction occurred in the column.

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