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Crystal Structures of Tropomyosin and Its Subunits, and Their Variation through Binding of Troponin

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Introduction

Tropomyosin discovered from rabbit skeletal muscle, characterized and crystallized in 1948[1], has been thereafter studied from various angles as a specific protein. Recently, this protein, however, has been found not only in the striated muscle, but also in smooth muscle, heart muscle of vertebrate and even in slime mold[10] and blood platelet[3].

The biological function of tropomyosin in the living muscle had never been elucidated for a long time until Ebashi[5] succeeded in fixing this protein to be a significant constituent of the so called "native tropomyosin" in the thin filament and to be an important mediator of Ca^{2+}-depending regulation in cooperation with troponin which is identified to be Ca^{2+} receptor site in myofibril on muscle contraction. Nowadays, it is evident that, in the thin filament, tropomyosin associates laterally with F-actin filament as two long chains consisting of end to end binding, and troponin binds the specific portion of tropomyosin with 400 Å period. As a biological function, tropomyosin, following the conformational change of troponin with Ca^{2+}-binding, shifts its position on to the periphery of the actin filament where probably it inhibits access of myosin to its actin-binding-site during relaxation[9].

It has been known that tropomyosin is to be separated into two subunits if it is analyzed through SDS polyacrylamide gel electrophoresis[14], one of which is named as α-component with 34,000 dalton and the other, β-component with 36,000 dalton peptide chain weight. And Commins and Perry[4] perfectly succeeded in the separation of them through CM-cellulose column chromatography in 8 M urea-containing buffer solution system. Then, Ookubo *et al.*[13] succeeded in getting the paracrystals of the subunits. While, Yamaguchi *et al.*[15] separated only one subunit of tropomyosin (SB–TM) corresponding likely to α-component through hydroxylapatite column chromatography, and then, succeeded in crystallizing itself and troponin-bound one. Caspar *et al.*[2] and Ooi *et al.*[11] also observed the troponin-bound tropomyosin crystal, however, β-component one and its binding capacity to troponin has never been investigated yet. In the present work, we investigated on the possibility of crystallization of the two subunits of tropomyosin, structural differences between crystals from both the subunits and the effects of troponin-binding on the respective crystal structure.

Materials and Methods

Preparation and purification of tropomyosin and troponin —— In this experiment, porcine

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longissimus thoracis and *biceps femoris* muscles resected within one hour after slaughter, were used as materials for preparation of the proteins. The tropomyosin was prepared from the crude tropomyosin fraction separated in the course of troponin preparation[6] and purifed as described by Hirabayashi[8], *i.e.*, crude tropomyosin was dissolved in 1 M KCl adjusted to pH 4.5 with 1 N HCl at 0°C, then tropomyosin was precipitated after 1 hour standing. The precipitate was collected by centrifugation at 10,000×g for 20 min, then, dissolved in 10 volume of water at pH 7.0 with 1 N NaOH added, and was left standing for 30 min. After the elimination of the insoluble matter was done by the centrifugation as above, the supernatant was fractionated three times with ammonium sulfate: at the 1st step, between 40–70%, at the 2nd step, 50–69% and at the last step, 53–60% saturations.

Each step was conducted after dialysis against 50 volume of deionized water kept under successively exchangings. Yield of the purified tropomyosin was 100 to 200 mg/kg of muscle. The obtained tropomyosin was preserved in the freezer at −20°C before use. In this case, tropomyosin solution was adjusted to pH 3.0 with 1 N HCl and 1 mM dithiothreitol was added for preventing tropomyosin from oxidizing the cysteine residue enclosed inside the molecule.

Separation of subunits from the purified tropomyosin —— Separation of subunits (α- and β-components) from tropomyosin purified as above was carried out according to the method described by Cummins and Perry[4], *i.e.*, both component fractions were obtained from CM-cellulose (Whatman CM 52, 1×12 cm) column chromatography of tropomyosin with a buffer system containing 8 M urea, 20 mM 2-mercaptoethanol, 50 mM sodium formate (pH 4.0) involving a linear gradient of NaCl from 0–0.2 M. Each fraction was collected and pooled at above 0.05 of the absorbance at 280 nm, and was dialyzed against 5 volume of 7.13 mM 2-mercaptoethanol solution exchanged successively for 2 days at 4°C, for the elimination of urea, and then the pH was adjusted to 3.0 with 1 N HCl, and it was stored frozen at −20°C.

SDS polyacrylamide gel electrophoresis —— Polyacrylamide gel electrophoresis in 1% of sodium dodecyl sulfate and 2-mercaptoethanol was carried out according to the method described by Greaser and Gergley[7].

Protein concentration —— Protein concentration was determined by the biuret method using bovine serum albumin as a standard.

Protein crystallization —— The solutions of the tropomyosin and its subunits were diluted or condensed using Centriflo membrane corn, type CF 25 (AMICON Co.) to about 5 mg/ml, and then, pH was once adjusted to 7.0 with 0.1 N NaOH. These solutions (1 ml) were placed in a 8 mm ID Visking tube and were dialyzed against 250 ml of crystallizing medium described below. The medium was stirred continuously with a magnetic stirrer at 2°–4°C during dialysis with the successive pH adjustments made until it reached 5.62, at 12 hours intervals. Some visible suspensoid appeared 1–3 days later inside the tube.

In the studies involving troponin, the troponin solution was also adjusted to pH 7.0 with the addition of 1 N NaOH first. For producing the crystals from tropomyosin or its subunits and troponin mixtures, two different procedures were adopted. Namely, in one procedure, the crystal suspension previously formed from tropomyosin or its subunits was well mixed with 0.5 ml of the troponin solution (5 mg/ml) and the mixture was further dialyzed against the crystallization medium. In another procedure, the solution of troponin and tropomyosin or its subunits premixed in the ratio of 1 : 2, was dialyzed against the crystallization medium. Crystals, however, were formed only in the former procedure, while in the latter, their formation was never realized even after several days' dialysis. Therefore, only the former procedure was adopted in this experiment.

The crystallization medium suitable for crystallizing tropomyosin and its subunits were investigated in advance.

As for the crystallization of tropomyosin, Ooi *et al.*[11] reported that it was possible for tropomyosin to be crystallized within the range of 0.1–0.4 M KCl, pH 5.1–5.9, above all, optimaly at 0.2 M KCl, pH 5.6. We also searched the optimal condition for the crystallization of tropomyosin and its subunits in the range of 0.15–0.25 M KCl, pH 5.5–5.7. As the result of it, we found out they could be crystallized in the same condition as tropomyosin, *i.e.*, they were optimaly crystallized through dialysis against the crystallization medium (0.20 M KCl, 10 mM Tris-maleate, pH 5.62) at 4°C. Under this condition, tropomyosin and its subunits were usually crystallized during dialysis overnight.

Electron microscopic observation —— Electron microscopic observations of the crystals were made after negative staining, *i.e.*, one drop of the crystal suspension concentrated by centrifugation (6,000×g, 10 min) was placed on a 200 meshed grid which was covered with a carbon-coated collodion film. After standing for one minute, the excess solution was absorbed from the side of the grid using a piece of filter paper and one drop of 1% uranyl acetate solution (0°C) was immediately added. After 10–20 seconds, the excess stain was removed and the grid was allowed to dry for 5 to 10 min. The samples were examined with JEM-100C electron microscope (Japan Electron Co.) at an accelarating voltage of 8 kV. Photographs were taken at direct magnification of 40,000 to 100,000 on Fuji electron image film.

Results

Separation of tropomyosin subunits —— The typical elution pattern of tropomyosin subunits separated through CM-cellulose column chromatography according to the method of Cummins and Perry[4] was shown in Fig. 1. The subunits were clearly separated and β-component was located in tubes 102–110 and α-component was in tubes 125–135. SDS polyacrylamide gel electrophoreses of the components corresponding to these peaks in the pattern revealed a difference in their band patterns (Fig. 2). Tropomyosin before separation into subunits, showed the double bands, whereas α-component showed only a fast-descending single band with 34,000 dalton and β-component, only a slow-descending single band with 36,000 dalton peptide chain weight.

Crystal structure of tropomyosin —— Tropomyosin could be readily crystallized. On the crystal and paracrystal of the tropomyosin, a lot of researches[15,2,11] have already been made, with reports published.

A typical electron micrograph of tropomyosin prepared in this study was shown in P–A1. As shown in the photograph, crystal structure of tropomyosin consisted of a pair of lines with approximate length of 220 Å and 180 Å, which were intersected to make a kite-shaped tetragon, namely, a sort of lattice consisted of two long sides and two short sides, and each side not being linear but undulate. If observed carefully, it would become evident that one side is constructed by two fibers. As pointed out by Ooi *et al.*[11], both the long and short sides may be made of double strands consisting of two fibers. The length of each side of lattice was measured on the micrograph in practice as to 20 lattices, and the averaged lengths were calculated. At the same time, a schematized reproduction was made in P–A2 and the dimensions of crystal lattice were summarized in Table 1. The length obtained by conjoining both long and short sides reached approximately 400 Å, which was in agreement with the length of the tropomyosin's monomer[8]. The acute angle formed by the adjacent short sides was 85° and that by the adjacent long sides was 61°.

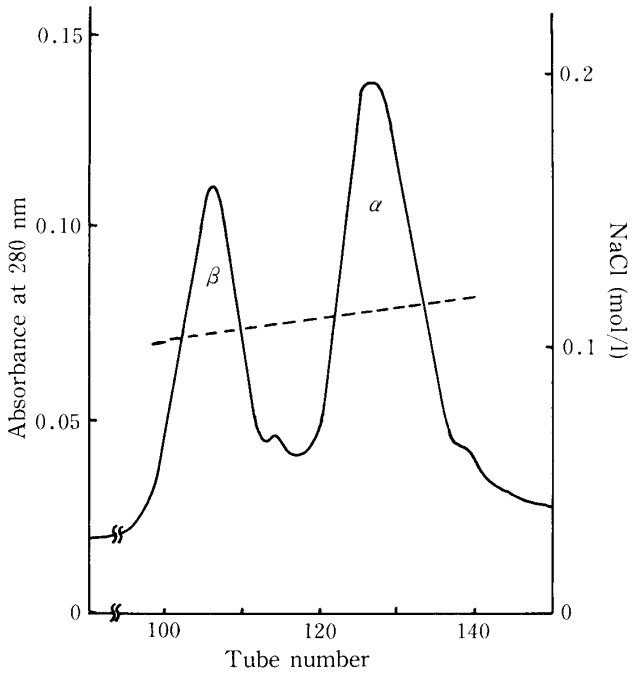


Fig. 1. A typical chromatogram on the separation of porcine skeletal muscle tropomyosin into two subunits (α- and β-component).
According to Cummins and Perry (1973)
Column: Carboxymethyl cellulose (Whatman CM 52), 1×12 cm.
Elution: Buffer; 8 M urea-containing 50 mM formate buffer, pH 4.0, 20 mM 2-mercaptoethanol.
Sample applied: 10 mg, flow rate: 5 ml/hour, NaCl-linear gradient (0–0.2 M).
Remark: Dotted line shows the NaCl concentration.

Crystal structure of α-component of tropomyosin subunits —— As mentioned above, α-component of tropomyosin subunits was also crystallized by the same procedure as the one described concerning tropomyosin itself. A typical electron micrograph was shown in P–B1. The crystal lattice consisted of pair of lines with the averaged length of 213 Å and 171 Å, which were intersected to make a kite-shaped tetragon like tropomyosin. The distance between the respective second intersecting points was 384 Å, which was shorter in comparison with that of tropomyosin. The angle formed by the intersection of the two short sides was 81° and that by intersection of the two long sides was 61°. The angle formed by the intersection of the short and long sides was 109°. The crystal pattern resembled that of tropomyosin crystal, whereas the crystal dimension was smaller either in the long side length or in the short side length, and the angle formed by the intersection of the short sides was 4° less, whereas the angle formed by the intersection of the short and long sides was 2° larger, in comparison with that of tropomyosin. The length of short side plus long side was also 16 Å shorter than that of tropomyosin. As a whole, compared with the lattice dimension of tropomyosin crystal, that of α-component crystal was a little smaller, as shown in Table 1. Besides, a characteristic feature of α-component crystal existed in the structure of the side itself, *i.e.*, it was quite evident from micrograph that both the short and long sides of crystal lattice consisted of single strand, not of double strands as in case of the tropomyosin crystal.

Crystal structure of β-component of tropomyosin subunits —— β-Component of tropomyosin subunits was readily crystallized by the same procedure as the one described concerning tropomyosin

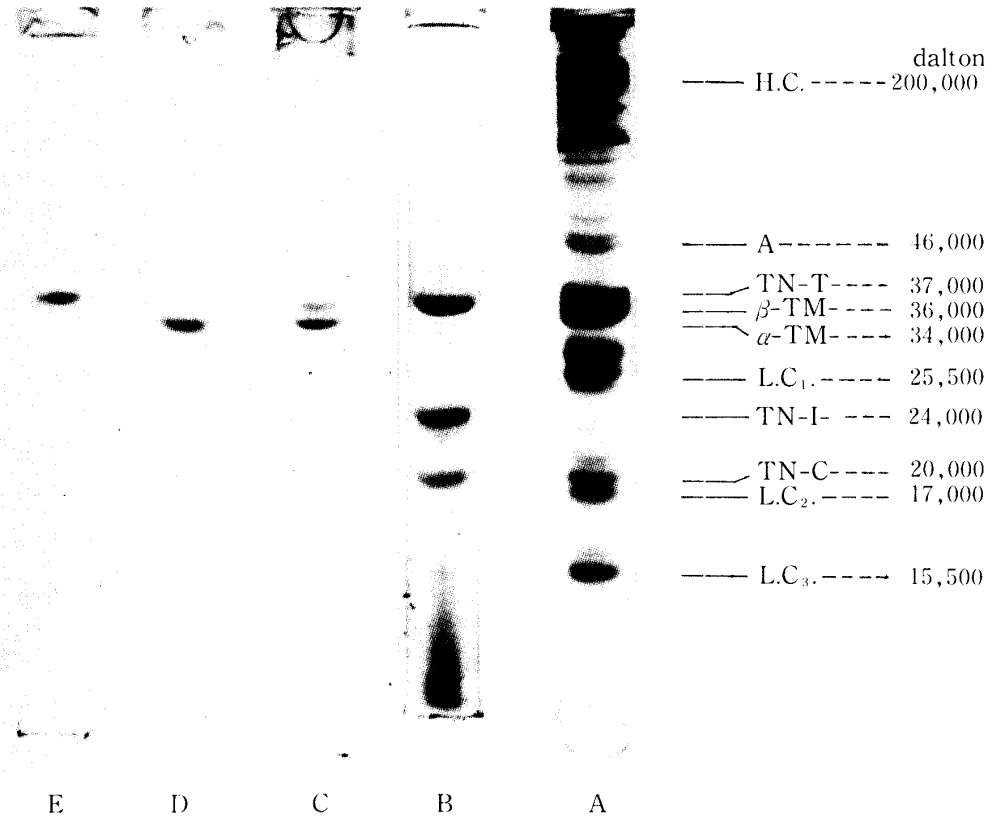


Fig. 2. SDS polyacrylamide gel electrophoretic patterns of the proteins prepared in this experiment. Electrophoreses were conducted with 10% acrylamide gels which were 10 cm in length, using the procedure described by Greaser and Gergley (1971).

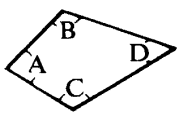
A: Myofibril from porcine skeletal muscle, B: Troponin, C: Tropomyosin, D: α-Component of tropomyosin, E: β-Component of tropomyosin.

Abbreviation:

H. C.: Myosin heavy chain, L. C_1, L. C_2, L. C_3: Myosin light chains, A: Actin, TN-T: Troponin-T, TN-I: Troponin-I, TN-C: Troponin-C, α-TM: α-Component of tropomyosin, β-TM: β-Component of tropomyosin.

and α-component. A typical electron micrograph was shown in P–C1. β-Component crystal also consisted of a kite-shaped lattice structure. However, the side constructing the lattice was made of single strand, and not double ones, likewise in case of α-component. In comparison with the lattice of α-component, the angle formed by the intersection of the short sides of the β-component crystal was larger. Namely, the tetragon shape of β-component was not so cute as that of α-component. The dimensions measured in practice were shown in Table 1 and the schematized reproduction

Table 1. Dimensions of crystal structures of tropomyosin and its subnuits

	Lattice length		Monomer length	Lattice angle			
	Long side	Short side					
	Å	Å	Å	A	B	C	D
Tropomyosin	220	180	400	85°	107°	107°	61°
α-Component	213	171	384	81°	109°	109°	61°
β-Component	210	174	384	85°	103°	103°	69°

based on them was shown in P–C2.

Crystal structure of reconstituted tropomyosin —— As the condition protecting SH-group from oxidation was adopted throughout the whole separation process of tropomyosin into subunits, the mixture of the equal amount of both the components was expected to reproduce the same crystal structure as that of the original tropomyosin. Then, we tried to crystallize the mixture. As the result of it, we could obtain the same crystal as that of the original tropomyosin (P–D1). From this fact, α- and β-components were confirmed not to be damaged by any oxidation of SH-group but to retain the adequate rebinding quality.

Crystal structure of troponin-bound tropomyosin —— As described before, the crystallization of premixture of troponin and tropomyosin or its subunits, was impossible, though it was unclear why such a result was obtained. So, it was inevitable to adopt the method of mixing troponin with tropomyosin crystal suspension previously prepared, dialyzing against the crystallization medium overnight. Electron micrograph of the tropomyosin crystal obtained by the method as mentioned above was shown in P–E1. The crystal lattice structure of troponin-bound tropomyosin was already different from that of tropomyosin itself and showed more square structure which consisted of double strands. When observed in more detail, two kinds of strands were paralleled alternatively to the vertical direction with a regularity, *i.e.*, one of them was a thick strand and another was two thin strands composed of the separated double strands with about 50 Å interval. The periodical spacing between the two kinds of strand was about 160 Å, while, to the horizontal direction, another kind of strand was paralleled. The strand was also double strands composed of both a waving strand repeating alternatively zig-zag form and a straight strand which was 200 Å as the periodical distance between the adjacent strands. The relation of these strands was schematically shown in P–E2. The angles at the intersection of these strand were in the range about 86°–94°, though they seemed to be nearly right angle.

Crystal structure of troponin-bound α-component of tropomyosin —— A micrograph of the crystal structure of troponin-bound α-component of tropomyosin was shown in P–F1.

The crystal lattice of troponin-bound α-component consisted of square tetragon which was clearly different from the kite-shaped form, being similar to that of α-component itself as described before. A schematized reproduction based on the dimensions which were measured in practice was also shown in P–F2. The strands to the vertical direction were composed of the repetition of the single and paralleling straight ones with the unequal spacing (165 and 215 Å); while to the horizontal direction, those were composed of the repetition of the single but zig-zag one with 170 Å spacing at the narrower portion or 218 Å spacing at the wider portion. At the intersecting points, the horizontal strands usually intersected at nearly right angle with the vertical strand inside the narrower spacing (165Å), whereas at the oblique angles which were 85° or 95°, inside the wider spacing (215 Å).

Crystal structure of troponin-bound β-component of tropomyosin —— The crystal structure of troponin-bound β-component of tropomyosin was so simple in comparison with those of troponin-bound α-component or troponin-bound tropomyosin as shown in P–G1. All the crystal lattices of it were composed of the rectangular form, not kite-shaped as in case of the β-component. And the lattice was constituted with the intersecting single strand like that of troponin-bound α-component, differing from that of troponin-bound tropomyosin. A schematized reproduction based on the dimensions measured in practice was also shown in P–G2. The strands to the vertical direction were composed of the repetition of the single and paralleling straight ones with the unequal spacing (175 and 210 Å) as in the troponin-bound α-component, while those to the horizontal direction were

composed of the repetition of the single and straight ones with almost equal spacing of 196 Å. And the strands to both the directions were intersected at nearly right angle each other.

Discussion

Mutual relationships among the crystal structures of tropomyosin, reconstituted tropomyosin, troponin-bound tropomyosin, troponin-bound α-component and troponin-bound β-component —— As mentioned above, the crystal structures of the intact tropomyosin were the same as that of reconstituted tropomyosin from the mixture of equal amount of α- and β-components. Either α- or β-component crystal lattice, however, was smaller than that of intact tropomyosin and the angle of intersection was also different each other. So, it was assumed that, when tropomyosin crystal was formed, α- and β-components were overlapped with a little slip at the intersection of lattice, as shown in Fig. 3 schematically. As to this point, models were reported by Cohen *et al.*[3] based on the X-ray diffraction analyses indicating that the intersection of lattice was not a point but a zone with some width.

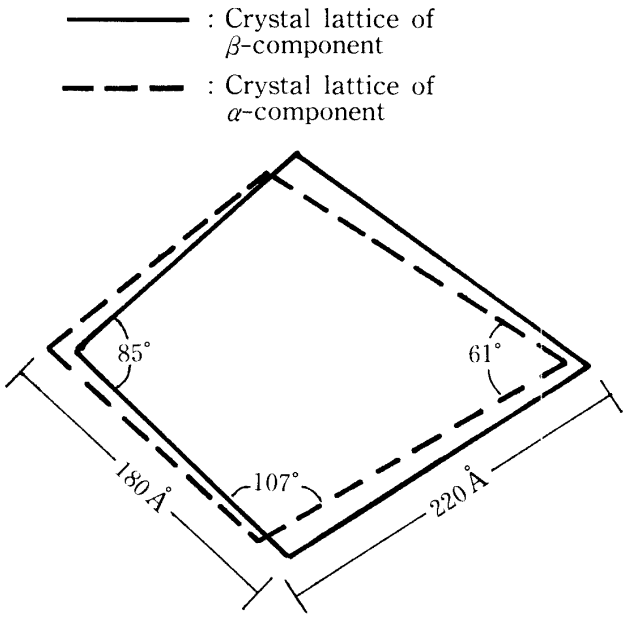


Fig. 3. A supposed organization of the crystal lattice of tropomyosin on the combination of two subunits.

It was well known that intact tropomyosin was readily precipitated together with Mg^{2+}, Ba^{2+} and Pb^{2+} to make paracrystals. When the paracrystals formed by them were observed through electron microscope, it was ascertained that they were composed of dark stripes with about 150 Å width and of light ones with about 250 Å width. Ookubo *et al.*[13] succeeded in making paracrystals of α- and β-components of tropomyosin, and reported that these paracrystals had 400 Å period in agreement with that of tropomyosin in this experiment. However, the crystal lattice length (monomer length) obtained from α- and β-components of tropomyosin showed a little shorter one. The reason why those lengths were shorter is not possible to be elucidated at present.

Yamaguchi *et al*[15]. succeeded in crystallization of SB–TM (single band tropomyosin), which was separated by hydroxylapatite column chromatography of tropomyosin in 0.2 M KCl, 0.01 M

Na-acetate, pH 5.6. This fragment of tropomyosin had 34,000 dalton peptide chain weight, which seemed to be corresponding to α-component. However, its crystal structure was considerably different from ours. When this point involving the findings about paracrystals was considered

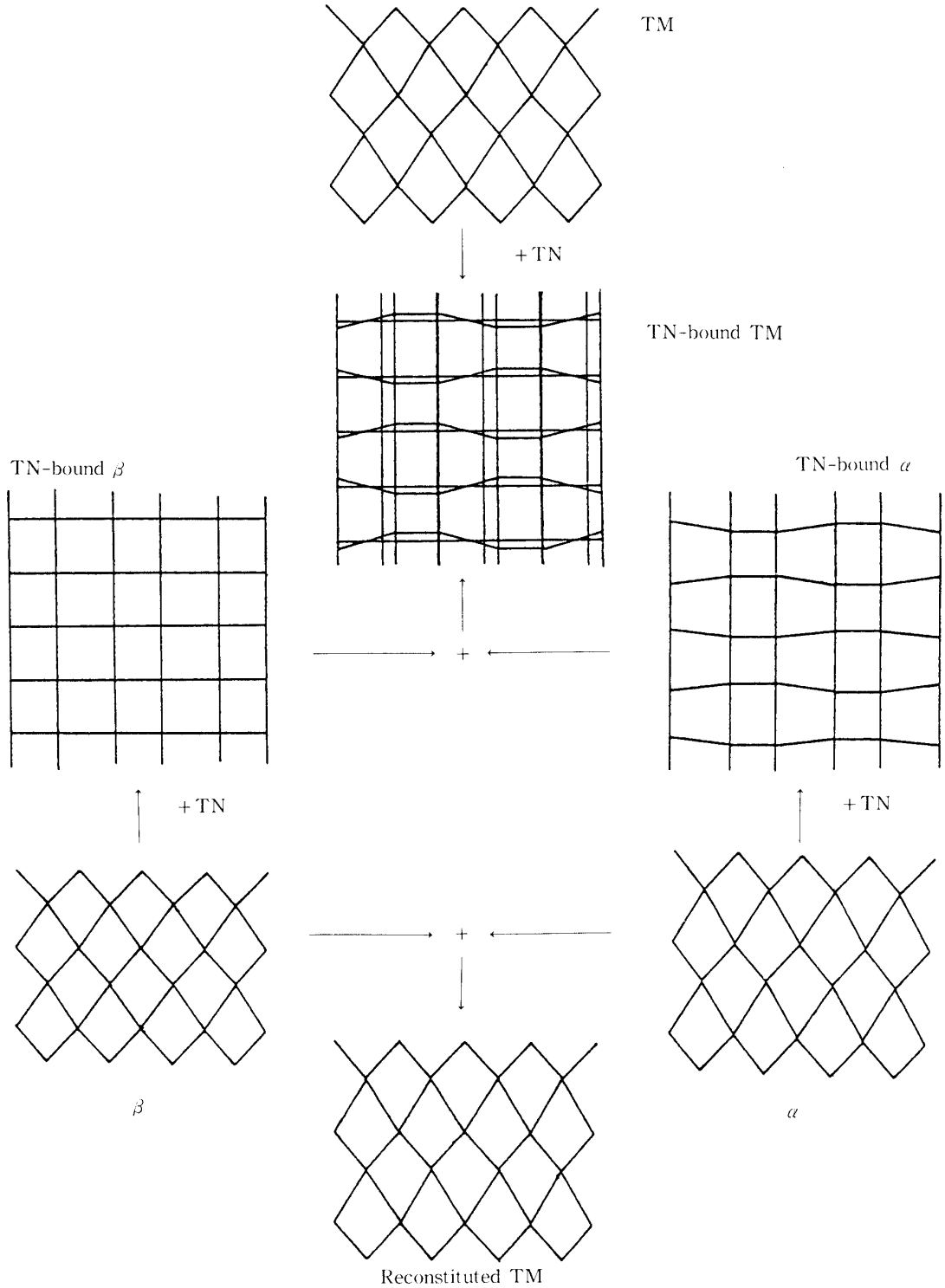


Fig. 4. A tentative draft elucidating the mechanism of crystal structure variation of tropomyosin through the binding of troponin based on the variation of subunits' crystal structure.
Abbreviation; TM: Tropomyosin, TN: Troponin, α: α-Component, β: β-Component.

SB–TM by Yamaguchi *et al*[15]. seems to be a matter a little different from the α-component of tropomyosin, because the separation of SB–TM was conducted at pH 6.9 paying little care for the oxidation of SH-group in tropomyosin molecule.

Cummins and Perry[4] reported α- and β-components could be separated by electrofocussing method to the lower peptide chains, named $\alpha 1$ and $\alpha 2$ from α-component, and $\beta 1$ and $\beta 2$ from β-component. Further, Ookubo[12] reported that $\alpha\alpha$ and $\alpha\beta$ type chains existed in α-component and $\beta\beta$ and $\alpha\beta$ chains, in β-component. The relations between the crystal structure and the lower subnits mentioned above should be investigated hereafter.

Relationship between the crystal structures of tropomyosin and its subunits and the variations through binding of troponin with them —— It was evident that when the crystals of tropomyosin and its subunits bound with troponin, the lattice structure was characteristically converted from the kite-shaped form to the rectangular tetragon. Further, the strands composing the lattices also seemed to be partially separated into two strands. The cause of such phenomena as above might be elucidated from the concept that α-and β-components of tropomyosin were intended to form the individual crystal structure through binding of troponin with them, respectively, as shown in Fig. 4 schematically.

Summary

In this study, the crystal structures of tropomyosin and its subunits (α- and β-components) from porcine skeletal muscle were investigated. And at the same time, the structural variations through the binding of troponin with them were surveyed using the electron microscope. The results obtained were as follows:

Tropomyosin and its subunits were crystallized easily under the same condition, that is, dialysis of those protein solutions against 0.2 M KCl, 0.01 M Tris-maleate buffer (pH 5.62) for 1–3 days at 4°C. The crystal structures obtained from those three sources were in mutual resemblance, one another, accompanied with the similar kite-shaped lattices.

It was evident that, however, the crystal lattices of α- and β-components were a little smaller than that of tropomyosin. Nevertheless, the trial to crystallize the equally amounted mixture of both the subunits succeeded in getting the same crystal structure as that of the original tropomyosin.

Next, the relationship between the crystal structures of tropomyosin and its subunits and the variations through binding of troponin with them were investigated. Tropomyosin or its subunits bound with troponin in advance were never crystallized under the condition adopted in this experiment. Therefore, troponin was bound with the crystals previously prepared from tropomyosin or its subunits.

The three kinds of crystal obtained as above, were all different from the ones before being bound with troponin, *i.e.*, the crystal lattices became of more rectangular form. However, the details of the lattices showed the following features; namely, the crystal structure of tropomyosin bound with troponin was basically composed of tetragon with 200 Å spacing and the lattices forming the tetragon consisted of double strands with about 50 Å interval.

The crystal lattice of α-component bound with troponin consisted of the repetition of the single and paralleling straight strands with the unequal spacing (165 or 215 Å) to the vertical direction, while to the horizontal direction, it consisted of the repetition of the single but zig-zag one with 170Å spacing at the narrower portion or 218 Å at the wider portion.

The crystal lattice of β-component bound with troponin consisted of the repetition of the

single and paralleling straight strands with the unequal spacing (175 or 210 Å) to the vertical direction, while to the horizontal direction, it was composed of the repetition of the single and straight strands with almost equal spacing of 196 Å.

Conclusively, it was evident that, when the crystals of tropomyosin and its subunits bound with troponin, the lattice structure was characteristically converted from the kite-shaped form to the rectangular tetragon. Further, the strands composing of the lattices also seemed to be partially separated into two strands. The cause of such phenomena as above might be elucidated by the concept that α- and β-components of tropomyosin were intended to form the individual crystal structure through letting troponin bind with them, respectively.

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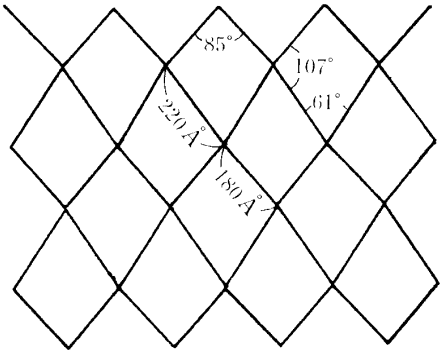
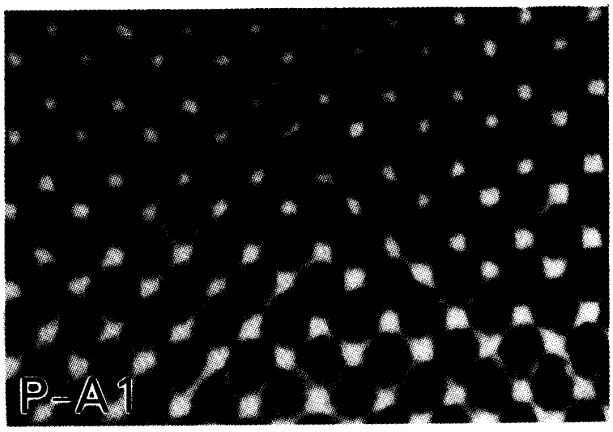
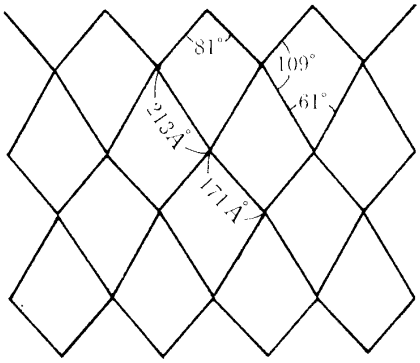
Explanation of photographs

P–A1. Electron micrograph of tropomyosin crystal taken at the direct magnification of 50,000.
P–A2. Schematized reproduction of lattice structure based on the measured dimensions in P–A1.
P–B1. Electron micrograph of α-component crystal of tropomyosin taken at the direct magnification of 50,000.
P–B2. Schematized reproduction of lattice structure based on the measured dimensions in P–B1.
P–C1. Electron micrograph of β-component crystal of tropomysoin taken at the direct magnification of 50,000.
P–C2. Schematized reproduction of lattice structure based on the measured dimensions in P–C1.
P–D1. Electron micrograph of the reconstituted tropomyosin crystal taken at the direct magnification of 40,000.
P–D2. Schematized reproduction of lattice structure based on the measured dimensions in P–D1.
P–E1. Electron micrograph of the troponin-bound tropomyosin crystal taken at the direct magnification of 100,000.
P–E2. Schematized reproduction of lattice structure based on the measured dimensions in P–E1.
P–F1. Electron micrograph of troponin-bound α-component crystal of tropomyosin taken at the direct magnification of 50,000.
P–F2. Schematized reproduction of lattice structure based on the measured dimensions in P–F1.
P–G1. Electron micrograph of troponin-bound β-component crystal of tropomyosin taken at the direct magnification of 50,000.
P–G2. Schematized reproduction of lattice structure based on the measured dimensions in P–G1.

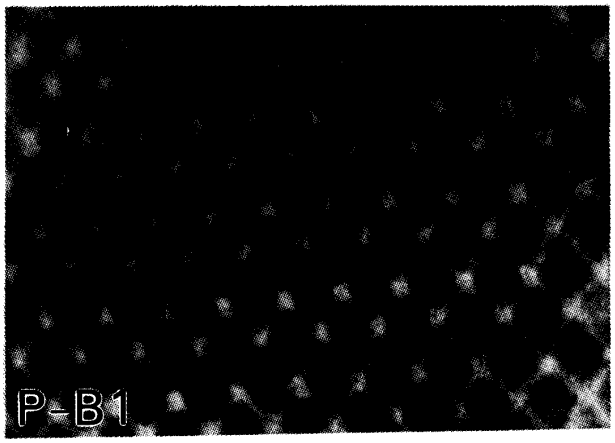


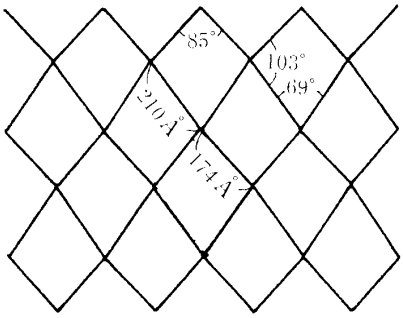
P-A2



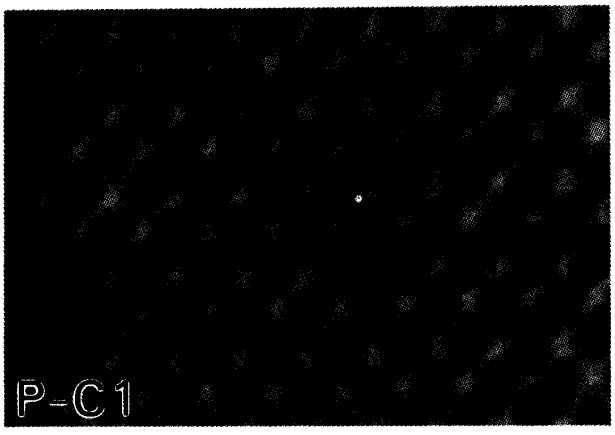


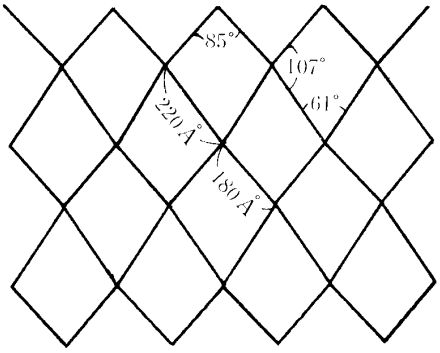
P-B2



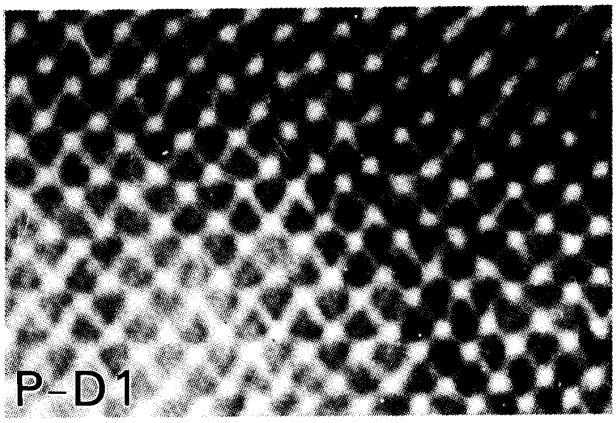


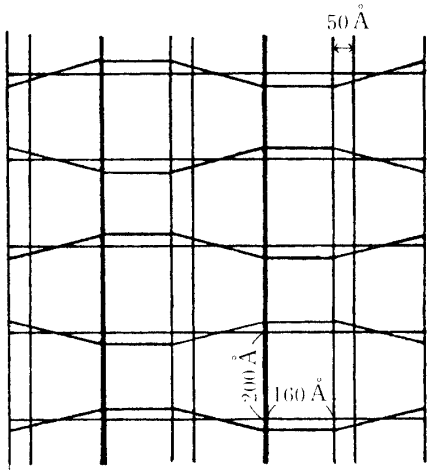
P-C2



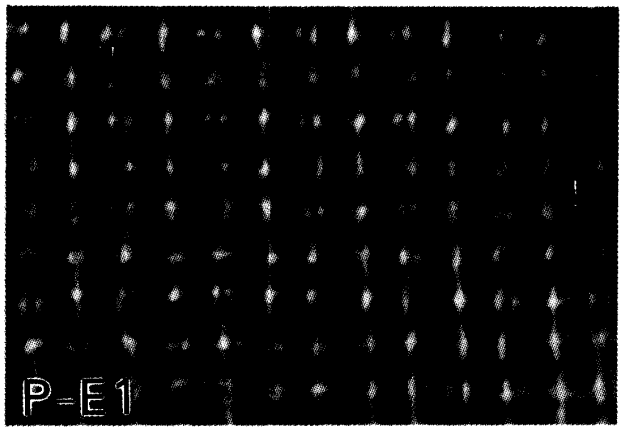


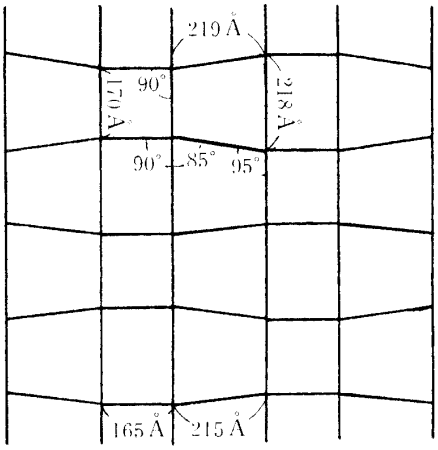
P-D2



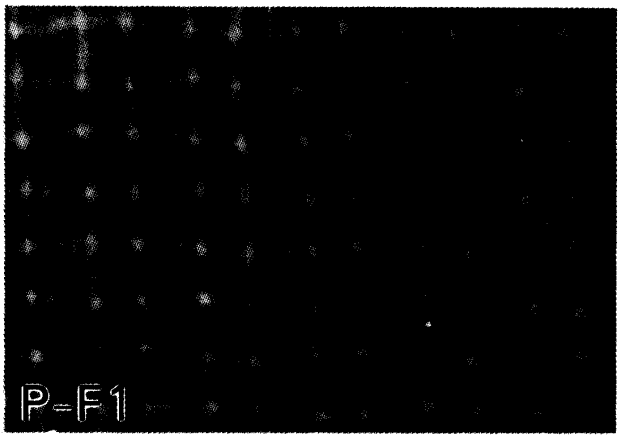

50 Å
200 Å
160 Å

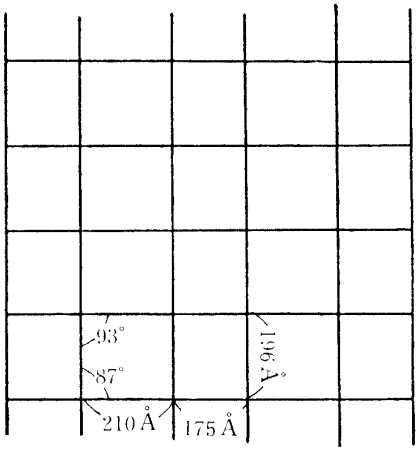
P-E2


P-E1

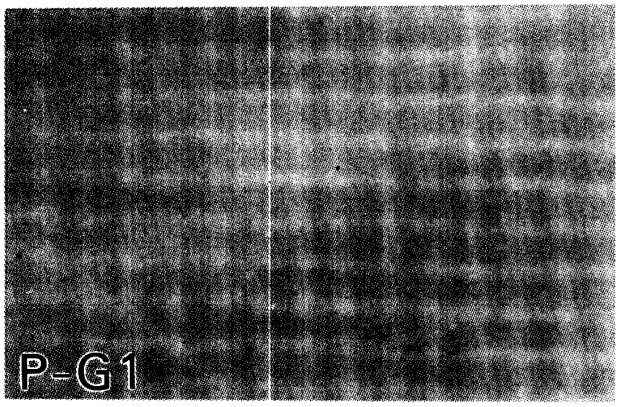

219 Å
90°
170 Å
218 Å
90°
85°
95°
165 Å
215 Å

P-F2


P-F1


93°
87°
196 Å
210 Å
175 Å

P-G2


P-G1