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# Subunit-Monomer Equilibrium of Maleylated Tropomyosin

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Tropomyosin, one of the muscle proteins, is a typical fibrous molecule constituted of two subunits, which has almost 100% of α-helix, forming a coiled-coil molecule. The molecule dissociates into subunits with a conformational change from α-helix to random coil when the lysine residues are maleylated. The modified molecule recovers its α-helical conformation with the increase in a salt concentration owing to the shielding effect of small ions added around negative charges. In order to clarify the problem whether or not any subunit of α-helical conformation can exist in solution, the circular dichroic spectra to estimate the α-helical content, and the sedimentation equilibrium to determine an apparent molecular weight of the protein were measured as a function of salt concentration. The results show that there is no single subunit molecule which has the α-helical conformation, and a conformational change from random coil to α-helix induces association of subunits.

KEY WORDS: Maleylated tropomyosin / Subunit-monomer equilibrium / Circular dichroism / Conformational change /

## INTRODUCTION

Tropomyosin, one of muscle proteins, is a typical fibrous protein constituted of two identical (or very similar) subunits, both having α-helical conformation to form a coiled-coil molecule. This molecule has a characteristic property to polymerize to a long fiber at a low salt concentration, and depolymerize into monomers by the addition of salt.[1] The molecule does not dissociate into subunits even in a very dilute solution due presumably to a strong hydrophobic interaction between two subunits as suggested by model building based on the amino acid sequence determined by Stone and Smillie.[2] However, when the lysine residues of tropomyosin are maleylated by the addition of maleic anhydride, the monomer molecule dissociates into subunits owing to the electrostatic repulsion which overcomes the hydrophobic interaction through a conformational change from α-helix to random coil.[3]

Since the electrostatic interaction may be weakened in the presence of salt ions, a restoration of α-helical conformation of maleylated tropomyosin is expected in the presence of excess salts. The α-helical conformation does recover with the increase in a salt concentration.[3] Now, a problem arises on whether any isolated subunit of α-helical conformation can exist or not in solution. The circular dichroic spectra to estimate the α-helical content, and the sedimentation equilibrium to determine an apparent molecular weight will clarify the present problem as will be described.

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## EXPERIMENTAL SECTION

**Materials** Tropomyosin was extracted from rabbit skeletal muscle according to the procedure of Ebashi *et al.*[4] and purified by the repeated cycle of isoelectric precipitation at pH 4.5 and fractionation by ammonium sulfate (*ca.* 47% saturation). The purified materials were free from contaminations such as troponin as judged by ultraviolet absorption spectrum and SDS-gel electrophoresis.

Maleylation was performed by the addition of maleic anhydride to a tropomyosin solution according to the procedure of Woods *et al.*[3] The extent of maleylation was determined spectrophotometrically by using the extinction coefficient for maleyl residue, $\varepsilon_{250}=3360$, reported by Butler *et al.*[5] The extent of maleylation thus determined was almost complete.

All the chemicals were of reagent grade, and potassium fluoride was selected as a neutral salt to be added because of its low extinction for ultraviolet absorption and high solubility in water.

**Methods** Circular dichroic spectra were measured using a spectropolarimeter, JASCO J-20, with a cell of 0.1 mm light path length in the wavelength range from 200 nm to 250 nm at 20°C. The mean residue molar ellipticity $[\Theta]$ (deg cm$^2$/decimol) is calculated according to the equation,

$$[\Theta]=[\Theta]_{obs}\times M/(10\times d\times C) \tag{1}$$

where $\Theta_{obs}$ is an observed ellipticity in degree, C is the protein concentration in g/ml, d is the path length, and M is the mean residue molecular weight, 128 for the maleylated tropomyosin.[3]

Measurements of sedimentation equilibrium were performed using a Spinco model E analytical ultracentrifuge equipped with an interference optical device at 20°C. The experimental runs were carried out at 14,290 rpm for 24 hours.

Interference fringes were measured with a Nikon profile projector.

The weight average molecular weight for a solution containing solutes of various molecular weights may be obtained by the following equation,

$$\bar{M}=\sum_i M_iC_i/\sum_i C_i=\frac{RT}{(1-\bar{v}\rho)\omega^2 r}\times\frac{d\ln C(r)}{dr} \tag{2}$$

where C(r) is a concentration at a position r, $\omega$ is the angular velocity of the rotor, $\rho$ is the density of the solution, and $\bar{v}$ is a partial specific volume of the protein (0.733 was used for maleylated tropomyosin[3]). We can obtain a concentration dependence of an apparent molecular weight from slopes of the plot of ln C(r) against $r^2$ by Eq. (2). However, the dependence is small for solutions which contain excess salts as described in the previous paper,[6] indicating that the virial coefficient of solutions of the present study is negligible. Therefore, apparent molecular weights from the Rayleigh interference patterns were calculated according to the conventional equation as follows,

$$M_{app}=\frac{2RT\times(C_b-C_a)}{(1-\bar{v}\rho)\omega^2\ C_0(r_b^2-r_a^2)} \tag{3}$$

where $r_m$ and $r_b$ are distances from the center of the rotor to the meniscus and the bottom of the solution in the cell, $C_m$ and $C_b$ are concentrations of the protein at the

meniscus and the bottom, respectively, and $C_0$ is the initial protein concentration. The apparent molecular weight thus obtained is that averaged over the concentration gradient in the cell.

The same solutions were used in both measurements, circular dichroic spectra and sedimentation equilibria.

## RESULTS AND DISCUSSIONS

As shown in Fig. 1, intact tropomyosin has a typical circular dichroic spectrum of $\alpha$-helix having two minima at 207 and 222 nm, respectively, while the spectrum of maleylated tropomyosin in the absence of salts does not show negative bands between 200 and 250 nm, indicating the destruction of $\alpha$-helical conformation on maleylation.[7] This may be accounted for by strong electrostatic repulsion between negative charges which are generated by substitution of amino groups on lysine residues to maleyl groups. The addition of guanidine HCl to the solution gives rise to a further increase in the ellipticity, suggesting that a conformation of maleylated tropomyosin in the absence of salts is not completely random, but has some ordered structure.

When the salt is added, the ellipticity decreases with the increase in the salt concentration and the two minima characteristic for $\alpha$-helix recover as illustrated in Fig. 1. That is, the $\alpha$-helical conformation of tropomyosin recovers with the addition of the salt due probably to weakening of electrostatic interactions in the presence of salt ions.

In Fig. 2, $[\Theta]_{222}$ as a measure of $\alpha$-helical content is plotted against the salt concentration. A sharp increase in the $\alpha$-helical content is observed near 0.5 M KF

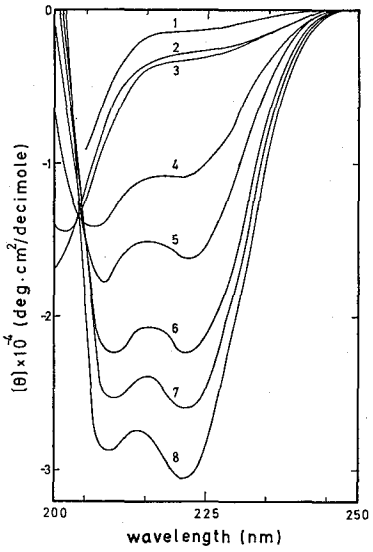


Fig. 1. Circular dichroic spectra of maleylated tropomyosin at various salt conditions. (1) in the presence of 6 M guanidine-HCl at 30°C. (2) in the absence of salt at 23°C. (3) 0.2 M KF at 23°C. (4) 0.6 M KF at 23°C. (5) 0.75 M KF at 23°C. (6) 1.5 M KF at 18°C. (7) 2.25 M KF at 23°C. (8) intact tropomyosin in 0.5 M KF at 20°C. The measurements were carried out at pH 6.9.

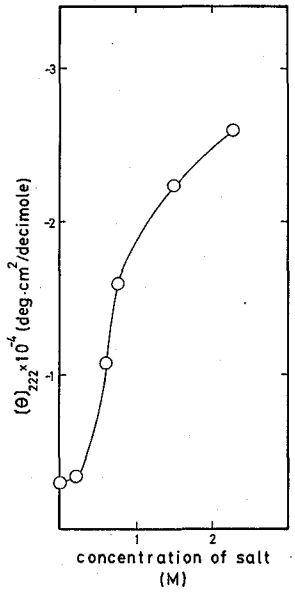


Fig. 2.

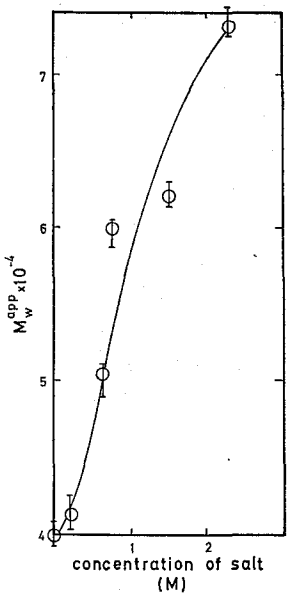


Fig. 3.

Fig. 2. Plot of $[\Theta]_{222}$ against the salt concentration.
Fig. 3. Plot of apparent molecular weight of maleylated tropomyosin against the salt concentration.

followed by a slower increment on a further increase in the salt concentration. Probably, the gain of the stability of $\alpha$-helix is critical depending on the decrease in electrostatic free energy induced by shielding of negative charges with small ions added.

Figure 3 shows the result of sedimentation equilibrium expressed by apparent molecular weights as a function of the salt concentration. On maleylation, a tropomyosin molecule dissociates into two constituent subunits, since the molecular weight of intact tropomyosin monomer, 74,000, decreases to that of subunits, 40,000. Since the concentration dependence of the apparent molecular weight was observed in the absence of salts, the determination of the molecular weight in a salt-free solution was performed by using Eq. (2),[6] instead of the approximate calculation by Eq. (3). The present value of the subunit obtained by sedimentation equilibrium experiments is somewhat larger than the calculated molecular weight according to the amino acid sequence of $\alpha$-tropomyosin calibrated with maleyl groups incorporated, 37,000. The discrepancy may be attributed to the specific volume for maleylated tropomyosin employed here, 0.733,[3] which might be a little higher than the real value, or to some effect of hydrated water molecules. Incidentally, the molecular weight obtained by physico-chemical methods appears to be always higher than the calculated value from the amino acid sequence, as is the present case. In any event, the result shows the dissociation of monomer into subunits on maleylation.

The apparent molecular weight of maleylated tropomyosin increases with the increase in the salt concentration (Fig. 3). (In the presence of salts, there was no concentration dependence of the apparent molecular weight as described before.) A significant increase in the molecular weight occurs between 0.2 M and 0.5 M KF, finally reaching the molecular weight of monomer, 74,000. This result indicates that

the maleylated tropomyosin cannot polymerize into a polymer despite the formation of the monomer molecule as intact tropomyosin at a high salt concentration. This may be ascribed either to a loss of polymerizability on modification of lysine residues or to depolymerizing effect in a high salt concentration. The process of the association of subunits seems to be in parallel to the change in $[\Theta]_{222}$ in Fig. 2.

Since we have the extent of the recovery of $\alpha$-helix and the apparent molecular weight of maleylated tropomyosin at the same salt concentration, the relation between the content of $\alpha$-helix and the degree of association may be analyzed quantitatively. The content of $\alpha$-helix is proportional to a fraction of the amount of residues in the $\alpha$-helical conformation, whereas the apparent molecular weight is the weight averaged quantity as $\sum_i M_i C_i / \sum_i C_i$. The present result shows that the system which we are dealing with is that of monomer-subunit equilibrium, *i.e.*, i=2. Therefore, a dimer fraction (X) in a salt concentration can easily be calculated from the apparent molecular weight experimentally obtained. Figure 4 shows the plot of $[\Theta]_{222}$ against the fraction X. Apparently, $[\Theta]_{222}$ is linearly dependent on X, indicating that the formation of $\alpha$-helix is proportional to the amount of monomer in solution. This result implies that there is no single subunit molecule which has $\alpha$-helical conformation, but those recovered $\alpha$-helix associate into monomer molecules.

Any rod-like protein molecule constituted of a single polypeptide chain which has almost 100% of $\alpha$-helical content has not been reported so far, although some synthetic homopolypeptides can exist in fully $\alpha$-helical form. For instance, typical fibrous proteins, tropomyosin and the rod part of myosin (L-meromyosin), are almost completely $\alpha$-helical but the molecules in solution are coiled-coils of two $\alpha$-helices, and single subunits exist only in denaturing medium. The present results just confirm the empirical situation that a fully $\alpha$-helical molecule of any protein does not exist.

The association into a monomer molecule by the addition of salts may be due to hydrophobic interactions between subunits, since, according to the amino acid sequence of tropomyosin,[2] hydrophobic residues align along the helical axis when $\alpha$-helix is formed. Subunits in random coil state originated from repulsion caused by negative charges on the chain will take the $\alpha$-helical conformation with the reduction of repulsive

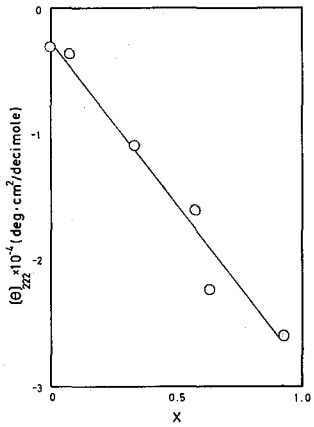


Fig. 4. Plot of $[\Theta]_{222}$ against the dimer fraction X of maleylated tropomyosin subunits.

energy on the addition of salts, when hydrophobic patches along the $\alpha$-helix tend to associate each other to form a stable monomer molecule. According to this explanation the amino acid sequence seems to be the inherent origin of the association of subunits.

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