A230Y Mutation of Actin on Subdomain 4 Is Sufficient for Higher Calcium Activation of Actin-Activated Myosin Adenosinetriphosphatase in the Presence of Tropomyosin−Troponin†

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ABSTRACT: To probe the mechanism by which Ca^{2+} activates muscle contraction through tropomyosin and troponin, we have produced mutant actins using *Dictyostelium discoideum*. We focused on the sequence 228−232 (QTAAS) that is located in subdomain 4 of actin, because the chimera actin in which this sequence was replaced by KAYKE showed not only poorer tropomyosin binding but also the unexpected "higher Ca^{2+} activation" [Saeki, K., et al. (1996) *Biochemistry 35*, 14465−14472]. We found that this higher Ca^{2+} activation is solely due to the A230Y mutagenesis. Because A230Y mutant actin showed normal tropomyosin binding, the higher Ca^{2+} activation is not the consequence of poorer tropomyosin binding. The significance of these results is discussed in view of a three-state model [McKillop, D. F., Geeves, M. A. (1993) *Biophys. J. 65*, 693−701].

The binding of calcium ions to troponin C triggers vertebrate skeletal muscle contraction through a series of interactions involving thin filament proteins, including tropomyosin and troponin (*1*), that regulate the interaction between actin and myosin that ultimately generates force by sliding (*2, 3*). However, the nature of these interactions and the precise mechanisms by which they control contraction are not completely understood. Three-dimensional cryoelectron microscopy has shown that tropomyosin binds to the inner domain of actin in the presence of Ca^{2+} ("on" state), which corresponds to subdomain 3 and 4 (*4*−*6*). Although electron microscopy of negatively stained specimens (*7, 8*) or ice-embedded specimens (*5, 6*) and the modeling based on X-ray scattering (*9*−*12*) have also given an indication of how tropomyosin is relocated to a higher radius in thin filaments in an "off" state, clear three-dimensional images of actin−tropomyosin−troponin have not yet been obtained with sufficiently high resolution to enable the precise binding site for tropomyosin in the "off " state to be established.

The primary structure of actin is highly conserved across species. However, *Tetrahymena* actin sequence is comparatively divergent (*13*) and cannot bind either tropomyosin or phalloidin (*14*). In addition to the variability observed between species at the N-terminus of the actin sequence, *Tetrahymena* actin also shows variability in residues 228−232, which are located in subdomain 4. Among actin-related proteins (Arps), insertion at 228/229 and deletion at 229−232 are observed (*15*). Though the general folding pattern of heat shock protein 70 (hsp70) is similar to that of actin, there is a large deletion: sequence 217−236 of actin is replaced by only three residues in hsp70 (*16*). These results suggest that mutation on the sequence 228−232 would not affect the folding of actin greatly. At the same time, this shows that the sequence 228−232 is characteristic for actin and is important for its function.

The actin chimera in which the *Dictyostelium* sequence was replaced by the corresponding sequence of *Tetrahymena* (228−232: QTAAS to KAYKE replacement) binds tropomyosin poorly as expected, but surprisingly, it shows a higher ratio of activation of the myosin S1 ATPase in the presence of tropomyosin−troponin and Ca^{2+} to that in the absence of tropomyosin−troponin than the wild-type actin (*17*). We refer to this effect as "higher Ca^{2+} activation". This result suggests that nonelectrostatic interactions may be important for both tropomyosin binding and calcium regulation of muscle contraction, because the QTAAS sequence contains no charged residues.

We report here more detailed experiments that establish that the A230Y mutation is solely responsible for higher Ca^{2+} activation. These findings may be explained by a allosteric/cooperative model such as a three-state model (*18*) but not by a simple steric block model.

MATERIALS AND METHODS

Plasmid Construction and Transformation of Dictyostelium Cells. Figure 1 shows the mutant actins produced in the present work. Mutations were introduced into the actin 15 gene by oligonucleotide-directed mutagenesis (*19*). In all mutants, Glu360 was changed to histidine to facilitate separation of mutant actins from the wild-type actin by HPLC on DEAE-5PW (*17*). The E360H mutant actin polymerizes normally and has normal calcium regulation (*17, 20*). The ribbon model in Figure 1 is based on the X-ray structure of

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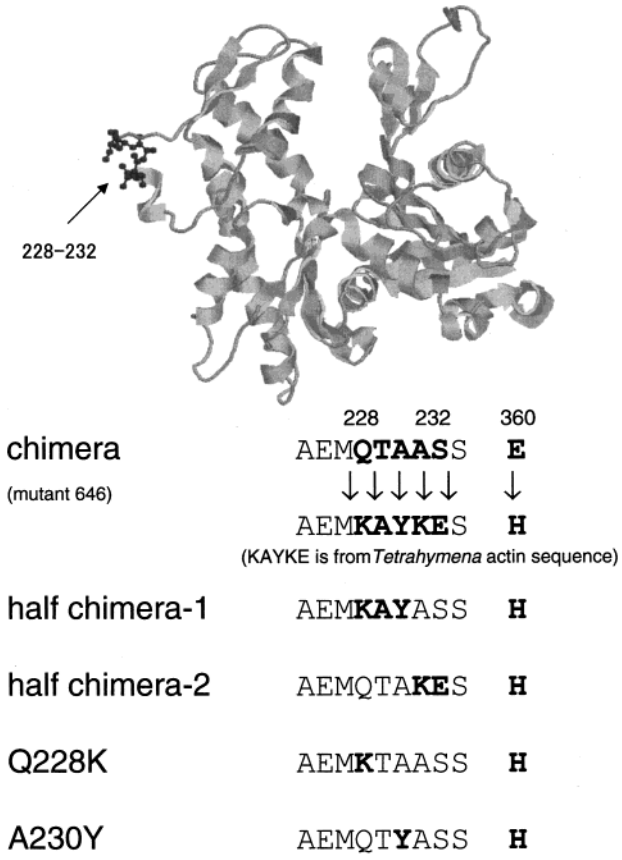

FIGURE 1: Ribbon model representation of rabbit skeletal actin (*21*) showing the positions of site-directed mutagenesis by ball-and-stick model and single-letter representation of the changes in the sequence of *Dictyostelium* actin. The crystal structure of *Dictyostelium* actin is almost the same as that of rabbit skeletal actin (Matsuura et al., *J. Mol. Biol.*, in press). The altered five residues in chimera mutant, which are the target of mutagenesis, are shown with ball-and-stick in the ribbon models and in boldface type in the sequences.

rabbit actin (*21*), but the atomic structure of *Dictyostelium* wild-type actin is almost the same as that of rabbit actin (Matsuura et al., *J. Mol. Biol.*, in press).

Transformation vectors were constructed by inserting the mutant actin 15 gene (*22*) into an integration vector B10Tp2′ (*23*) as described (*24*). Transformation vectors were introduced into *Dictyostelium* cells by electroporation (*25*). Transformed cells were selected by culturing them in the presence of G418 (neomycin analogue) as described (*17, 26*). Cells were cloned twice by picking them from a colony visible on a culture dish and plating them on a new dish. The expression level of mutant actin in these cloned cells was estimated by two-dimensional gel electrophoresis (*27, 28*), because all types of mutation described in this paper altered the isoelectric point of actin.

Preparation of Proteins. Transformed *Dictyostelium* cells were used to purify both mutant and wild-type actins as described (*17, 26*). Myosin was prepared from rabbit skeletal muscle (*29*). S1[1] (myosin subfragment 1) was obtained by digesting myosin with chymotrypsin following the procedure described previously (*30, 31*). Tropomyosin and tropomyosin−troponin were prepared from rabbit skeletal muscle as described by Ebashi et al. (*32*) and Ebashi and Ebashi (*33*), respectively.

Assay of Tropomyosin-Binding Ability. The tropomyosin-binding ability was examined by the method described previously (*17*). Briefly, the various concentrations of tropomyosin were mixed with a fixed amount (5 μM final) of actin. The mixtures were incubated for 2 h at 25 °C in 150 mM NaCl, 10 mM imidazole hydrochloride (pH 7.0), and 5 mM $MgCl_2$. The mixtures were then centrifuged at 360000*g* at 25 °C in a Beckman TL-100 ultracentrifuge.

The resulting pellets were resuspended in the same buffer in volumes equal to those of the supernatants, and then aliquots of both supernatants and suspended pellets were subjected to SDS−PAGE. The amount of tropomyosin bound to actin was quantified by densitometry of the stained bands as previously (*17*). By use of KaleidaGraph the dissociation constant (K_{app}) was determined by fitting the data to (*34*)

$$\upsilon = (n[\mathrm{TM}]^{\alpha}/K_{app}{}^{\alpha})/(1+[\mathrm{TM}]^{\alpha}/K_{app}{}^{\alpha})$$

where [TM] and α denote the molar concentration of tropomyosin and the Hill coefficient, respectively.

To measure tropomyosin binding in the presence of troponin, the various concentrations of tropomyosin−troponin were mixed with a fixed amount of actin (5 μM final). The mixtures were incubated for 2 h at 25 °C in 150 mM NaCl, 10 mM imidazole hydrochloride (pH 7.0), 5 mM $MgCl_2$, and 0.1 mM $CaCl_2$ or 0.2 mM EGTA.

ATPase Activity Measurement and Other Procedures. SDS gel electrophoresis was done on 12.5% polyacrylamide slab gels (*35*) and gels were stained with Coomassie Brilliant Blue. Two-dimensional electrophoresis was carried out by the method of O'Farrell (*27*) and Mikawa et al. (*28*). The first dimension (isoelectric focusing) was performed in the presence of 9 M urea. A 10% (w/v) polyacrylamide gel was used for the second dimension. Gels were stained with Coomassie Brilliant Blue.

Actin activation of S1-ATPase activity was measured as described previously (*17*). Briefly, the reaction mixture contains 50 mM KCl, 10 mM imidazole hydrochloride (pH 7.0), 5 mM $MgCl_2$, 1 mM ATP, 50 μM $CaCl_2$ or 0.2 mM EGTA, 0.87 μM S1, 14.3 μM actin, 4.4 μM tropomyosin−troponin, and 6.3 μM phalloidin. In the absence of tropomyosin−troponin, ATPase activities were measured in 50 mM KCl, 10 mM imidazole hydrochloride (pH 7.0), 5 mM $MgCl_2$, 1 mM ATP, 0.87 μM S1, 14.3 μM actin, and 6.3 μM phalloidin. In the presence of tropomyosin, the mixture contained 4.4 μM tropomyosin instead of tropomyosin−troponin. All reactions were carried out at 25 °C. Phosphate assays were done by the malachite green method (*36*). Phalloidin was added to ensure the polymerization of actin but did not affect the results.

Protein concentration was measured by absorbance at 280 nm or by the Bradford method (*37*) according to Read and Northcote (*38*), calibrated with ultraviolet absorbance unless otherwise stated. The values of absorbance of actin, tropomyosin, and tropomyosin−troponin were assumed to be 11.1 cm^{-1} (1%), 2.9 cm^{-1} (1% at 278 nm), and 3.8 cm^{-1} (1% at 278 nm), respectively (*39, 40*).

RESULTS

Tropomyosin Binding of Half-Chimera and Point Mutant Actins. Two half-chimera actins and two point mutants were

[1] Abbreviations: S1, myosin subfragment 1; EGTA, ethlene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

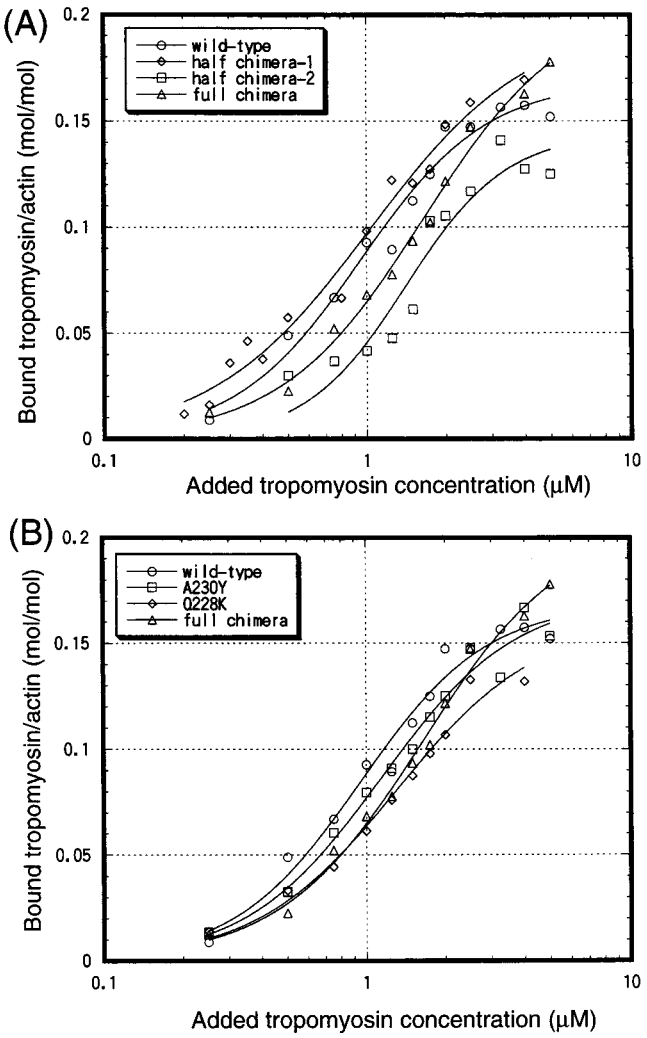

FIGURE 2: Tropomyosin-binding ability of (A) two half-chimera mutant actins or (B) two point mutant actins was compared with that of wild type or full chimera mutant. A final concentration of 5 μM wild-type actin (○), chimera actin (△), half-chimera 1 actin (◇ in panel A), half-chimera 2 actin (□ in panel A), Q228K (◇ in panel B), or A230Y (□ in panel B) was mixed with various concentrations of tropomyosin and incubated for 2 h at 25 °C in 5 mM $MgCl_2$, 10 mM imidazole hydrochloride (pH 7.0), and 150 mM NaCl. The mixture was ultracentrifuged. The supernatant and the pellet were analyzed by SDS−PAGE.

produced by protein engineering as shown in Figure 1. To achieve good separation from wild-type actin by DEAE-5PW column chromatography, the neutral mutation (E360H) was also introduced and found to be effective. All mutant actins were expressed and purified as described (*17*), and all showed a separate spot distinguishable from the wild-type actin in two-dimensional gel electrophoretic patterns.

Figure 2A shows that the half-chimera 2 actins bound to rabbit skeletal tropomyosin weakly, whereas the tropomyosin binding of half-chimeras-1 was normal. It was also confirmed that the chimera mutant binds tropomyosin weakly (Figure 2A). Figure 2B shows the tropomyosin binding to two point mutants: Q228K binds tropomyosin less strongly than A230Y, which shows almost same binding as the wild-type actin. In all cases, binding curves reached a plateau at the level of about 0.16, which approximately corresponds to 7:1 stoichiometry of actin to tropomyosin determined by electron microscopy (*41*). The apparent dissociation constants of wild-type actin and mutant actins obtained from the tropomyosin-binding experiments are tabulated (Table 1). The Q228K, half-chimera 2, and full chimera mutants showed significantly lower affinity for tropomyosin. However, they bind an almost stoichiometric amount of tropomyosin under the conditions of ATPase assay, because the added concentration of tropomyosin was several times higher than K_{app}.

Table 1: Apparent Dissociation Constants of Wild-Type and Mutant Actins as Determined by Cosedimentation with Tropomyosin

actin	sequence	K_{app}[a] (μM)
wild type	QTAAS	0.95 ± 0.09[b]
half-chimera 1	**KAY**AS	1.03 ± 0.17
A230Y	QT**Y**AS	1.13 ± 0.12
Q228K	**K**TAAS	1.30 ± 0.20[c]
half-chimera 2	QTA**KE**	1.41 ± 0.21[d]
full chimera	**KAYKE**	1.65 ± 0.17[d]

[a] The statistical significance of the difference in K_{app} was examined according to Numerical Recipes in C (*58*). [b] Error of estimation of K_{app}. [c] Larger than that of the wild type (confidence level of 93%). [d] Larger than that of the wild type (confidence level of 95%).

In the presence of troponin all actins showed the saturating binding of tropomyosin at the tropomyosin concentration of 2 μM irrespective of Ca^{2+} concentration. This also ensures that all types of actin bind tropomyosin−troponin fully under the conditions of ATPase assay, which was done by adding 4.4 μM tropomyosin−troponin.

Ca^{2+} Activation of S1-ATPase in the Presence of Tropomyosin−Troponin. Figure 3 shows the ability of the mutant actins to activate the myosin S1-ATPase under four conditions, i.e., pure actin, in the presence of tropomyosin, and in the presence of tropomyosin−troponin, either with or without Ca^{2+}. Without regulatory proteins, all of the mutant actins activated the S1-ATPase to the levels comparable to those observed with wild-type actin. In the presence of tropomyosin, the activation by actin from half-chimera 2 or Q228K was significantly lower than that by other mutants or the wild-type actin. It is interesting that these two mutant actins showed somewhat lower activation in the absence of tropomyosin. When both tropomyosin and troponin were added in the absence of Ca^{2+}, the activation by every actin mutant was suppressed in the same way as wild-type actin, indicating that the inhibition of the S1-ATPase by tropomyosin−troponin from rabbit skeletal muscle was functioning normally. However, in the presence of both tropomyosin−troponin and Ca^{2+} (solid bars in Figure 3), there were distinctive differences between the various mutants: The Ca^{2+} activation of the myosin S1-ATPase observed for the half-chimera 1 actin was almost 3 times higher than that for the wild-type actin, whereas that for the half-chimera 2 was similar to that for wild type. Thus the half-chimera 1 has retained the property of higher Ca^{2+} activation observed with the full chimera.

To establish the contributions made by individual residues in the half-chimera 1 to the higher Ca^{2+} activation, point mutants Q228K and A230Y were investigated. Because the actin with T229C mutation was similar to the wild-type actin (data not shown), it was anticipated that the introduction of K or Y would induce the higher Ca^{2+} activation. The A230Y mutant was found to show the higher Ca^{2+} activation as observed with the full chimera, whereas Q228K actin behaved as the wild-type actin. These results indicate that

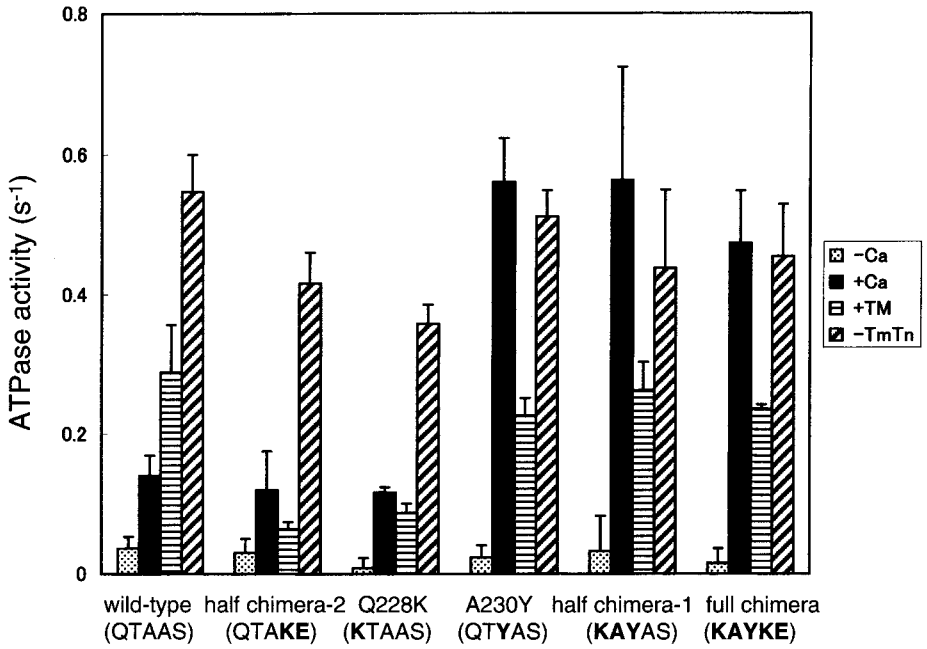

FIGURE 3: Activation of myosin S1 ATPase by various actins under the four conditions, i.e., pure actin (hatched bars), in the presence of tropomyosin (horizontally striped bars), and in the presence of tropomyosin−troponin, either with (solid bars) or without (dotted bars) Ca^{2+}. Actin activation of S1-ATPase activity was measured in the reaction mixture containing 50 mM KCl, 10 mM imidazole hydrochloride (pH 7.0), 5 mM $MgCl_2$, 1 mM ATP, 0.87 μM (0.1 mg/mL) S1, 14.3 μM (0.6 mg/mL) actin, and 6.3 μM (5 μg/mL) phalloidin. The added concentration of tropomyosin or tropomyosin−troponin was 4.4 μM. The added concentrations of $CaCl_2$ and EGTA were 50 and 200 μM, respectively. All reactions were carried out at 25 °C. Error bars indicate standard deviations ($n = 4$).

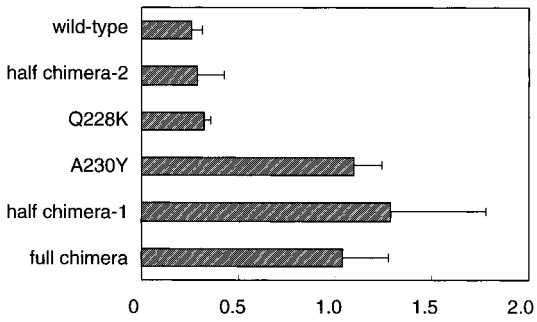

FIGURE 4: Normalized ATPase activation by various types of actin. To examine the effect of mutagenesis on the regulatory mechanism by tropomyosin−troponin, the ratio of activation of myosin S1 ATPase by actin in the presence of tropomyosin−troponin and Ca^{2+} to that in the absence of tropomyosin−troponin was calculated and shown as normalized ATPase activity. All mutant actins that have Ala230-to-Tyr mutation showed higher normalized ATPase activity (higher Ca^{2+} activation) and we call them Tyr mutants.

introduction of tyrosine at position 230 on subdomain 4 is sufficient for the higher Ca^{2+} activation.

To examine the extent of higher Ca^{2+} activation, the ratio of the activation of myosin S1 ATPase by actin in the presence of tropomyosin−troponin and Ca^{2+} to that in the absence of tropomyosin−troponin was calculated (Figure 4). This ratio (normalized ATPase activity) represents the effect of mutagenesis on the regulatory mechanism through the interaction of actin with tropomyosin−troponin, because the effect of mutagenesis on the activation by pure actin is eliminated by normalization. The mutant actins can be classified into two groups: A230Y, half-chimera 1, and chimera show 2−3 times higher normalized activity than the wild type, whereas half-chimera 2 and Q228K show similar normalized activity to that by the wild-type actin. Because the common feature of the former group is that the residue 230 was changed to tyrosine, we call them Tyr mutants. This indicates that the region around the residue 230 plays an important role in Ca^{2+} activation by tropomyosin−troponin.

DISCUSSION

Tropomyosin Binding of Actin Mutants. It has been proposed that charged amino acid residues are important for actin−tropomyosin interaction in both the "on" and "off" states. The distribution of charged amino acid residues along tropomyosin molecules shows 14 repeats (*42*−*44*). Elimination of two charged residues from a *Dictyostelium* actin (K238A/E241A) results in poorer tropomyosin binding (*17*). Chemical modification studies suggested that Arg95 (*45*), Lys238 (*46*), and Lys336 (*47*) of actin are involved in tropomyosin binding and that Lys61 (*48*) is important for the calcium-mediated regulation of the actin−myosin interaction. However, it has been known that the affinity of tropomyosin for actin decreases at very low ionic strength and tropomyosin−troponin can be extracted from muscle mince (*1*). This suggests that actin−tropomyosin interaction is not solely electrostatic.

There are a large number of hydrogen-bond-forming residues such as Ser, Asn, Thr, and Gln on the surface of the tropomyosin molecule (*44*). The hydroxyl groups of the QTAAS sequence on actin may form hydrogen bonds with tropomyosin that supplement the electrostatic interactions. The large charged side chains of Lys or Glu introduced at the first, fourth, or fifth positions of the pentapeptide (Q228K, half-chimera 2, full chimera) might disturb the interaction between actin and tropomyosin by decreasing the number of hydrogen bonds. It is interesting that the negative effect of Q228K mutation on tropomyosin affinity is dampened by simultaneous introduction of tyrosine in the half-chimera 1, which shows normal tropomyosin binding. It should be noted that the difference in tropomyosin affinity per se would

not affect the ATPase activation: an almost stoichiometric amount of tropomyosin was bound to every mutant actin, because tropomyosin concentration under the assay conditions was several times higher than K_{app} (Table 1).

Activation of Myosin ATPase by Mutant Actins. Though there seem to be slight variations in S1-ATPase activation by the different actins without tropomyosin−troponin, all of the *Dictyostelium* mutant actins activated ATPase normally. This activation was suppressed by rabbit skeletal tropomyosin−troponin in the absence of Ca^{2+}. This indicates that the basic process of actin activation and the inhibitory process in mutant actin systems are indistinguishable from that of the wild-type one. All actins with the A230Y mutation (Tyr mutants) showed higher Ca^{2+} activation. This indicates that only the Ca^{2+} activation process is appreciably affected by Tyr mutation. The variation in ATPase activation by different actins tends to be augmented by the addition of tropomyosin. The half-chimera 2 and Q228K actin showed significantly lower activation than other actins in the presence of tropomyosin but the difference is not so significant without tropomyosin. It is interesting that the tropomyosin affinity of these two mutants is lower.

Biochemical and electron microscopic studies on actin suggest the existence of interdependence between the C-terminus, the nucleotide-binding cleft, the bound metal, and DNase I binding loop (*49*−*51*). Feng et al. (*52*) reported that the binding of myosin S1 to actin changed the fluorescence of pyrene bound to the residue 265 in the inner domain (subdomain 4), but myosin mainly binds to the outer domain of actin (*4*, *53*, *54*) far from the subdomain 4. The atomic structure of the main chain of the half-chimera 1 is almost the same as that of the wild type as determined by X-ray crystallography, and the conformational changes due to the mutagenesis are restricted to the local side-chain conformation (Matsuura et al., *J. Mol. Biol.*, in press). Also, the predictions indicate that no appreciable changes in the secondary structure would occur by the introduction of mutagenesis into the sequence of QTAAS (228−232). Therefore, it is plausible that the effect of mutagenesis on the activation of myosin ATPase is not due to the structural changes of actin per se but through the changes in the interaction of actin with tropomyosin−troponin. Because Tyr mutants show normal activation in the presence of tropomyosin only, the role played by troponin may be more important.

It is possible to interpret our data according to a three-state model (*18*, *55*). We propose that actin from Tyr mutant favors an "open state" and push the equilibrium from a "closed state" toward an "open-state". This kind of higher Ca^{2+} activation cannot be explained by the simple steric blocking. Thus, the combination of the steric blocking (*9*−*11*, *56*) and an allosteric/cooperative model such as a three-state model is needed to explain the results of our mutagenesis experiments and many structural studies on thin filaments.

The increase of the intracellular concentration of Ca^{2+} causes a conformational change in troponin (C + I) (*6*), which is transmitted to actin through tropomyosin and/or troponin T. The local changes in the side-chain conformation caused by introduction of A230Y mutagenesis may alter the interactions between actin and tropomyosin and/or troponin. By modulating such interactions, Tyr mutant actin would favor an "open state". The conformation of the wild-type and Tyr mutant actins should be almost the same in the absence of Ca^{2+}. However, an equilibrium constant K_T (between "open state" and "closed state") of Tyr mutant actins may be larger than that of wild-type or "non-Tyr" mutant ones so that an "open state" is much more favored in the presence of Ca^{2+}.

In summary, the introduction of Tyr replacing Ala230 induces higher Ca^{2+} activation. The higher Ca^{2+} activation is not the consequence of poorer tropomyosin binding, because A230Y mutant actin binds tropomyosin normally. The introduction of charged residues such as Lys or Glu in the first or last two positions of pentapeptide (QTAAS) and/or exclusion of Gln or Ser, which can form hydrogen bonds, may be related to poorer tropomyosin binding. The higher Ca^{2+} activation by Tyr mutant actin may be caused by favoring an "open state". These mutant actins will help to elucidate the regulatory mechanism of muscle contraction.

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