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Biochemistry

Site-directed mutations of *Dictyostelium* actin: Disruption of a negative charge cluster at the N terminus

(*in vitro* motility assay/actin-activated myosin ATPase/actin–myosin interaction)

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ABSTRACT **Aspartic acid residues in the N-terminal negative charge cluster of *Dictyostelium* actin were replaced with histidine residues by site-directed mutagenesis of the actin gene. The mutant actins were expressed in *Dictyostelium* cells and were purified to homogeneity by HPLC. Functional properties of the mutant actins were compared with those of the wild-type actin. (*i*) *In vitro* assays of the sliding movement of actin filaments driven by myosin showed that the movement was slowed by the mutations. (*ii*) The mutations diminished the actin-activated ATPase activity of myosin in such a way that the maximum turnover rate at infinite actin concentration (V_{max}) dropped sharply without an appreciable change in the apparent affinity of actin and myosin (K_{app}). These results indicate that the N-terminal negative charge cluster of actin is essential for the ATP-dependent actin–myosin interaction.**

The N-terminal region of actin (residues 1–4) has a cluster of acidic residues irrespective of actin species (1–4). The three-dimensional structure of actin deduced from x-ray crystallography (5) has revealed that the cluster is spatially very close to another conserved cluster of acidic residues at the C-terminal region (residues 361–364). These N- and C-terminal regions form an unusual area rich in negative charges that may contribute to protein–protein interactions. In fact, chemical crosslinking by a zero-length crosslinker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, which detects contact sites of amino and carboxyl groups, has shown that the highly charged N- and C-terminal regions are in contact with various actin-binding proteins including myosin (6–11).

The myosin head is in contact with the charged N- and C-terminal regions of actin at least in the absence of ATP—i.e., in the rigor complex (6, 7). However, it seems that the contact does not contribute much to the binding energy of the rigor complex since an antibody raised against the N terminus of actin does not inhibit the actin–myosin association in the absence of ATP (12). On the other hand, in the presence of ATP, the same antibody inhibits the actin–myosin association, causing loss of activation of myosin ATPase activity (13). The result implies that the N-terminal segment is involved in the actin–myosin interaction during the ATPase cycle, although an allosteric effect by the antibody cannot be excluded.

The best way to understand the functional role of this unique area on the actin molecule is to replace these acidic residues and observe the consequences. Recent progress in recombinant DNA techniques enables one to replace virtually any residue in a protein. *Dictyostelium* cells have been used as host cells for genetic manipulation of contractile and cytoskeletal proteins such as myosin (14, 15) and α-actinin (16). Although it has been reported that actin expressed in *Escherichia coli* can be purified as a functional protein after detergent treatment (17), we preferred using *Dictyostelium* cells to express recombinant actins because an easier and milder purification procedure is possible for the latter system.

In this paper, mutations were introduced into the actin 15 gene of *Dictyostelium discoideum* so that the N-terminal sequence of the *Dictyostelium* actin 15 Asp-Gly-Glu-Asp- (4) was altered to His-Gly-Glu-Asp- and His-Gly-Glu-His-. These two mutant actins, D1H (replacement of Asp-1 with His; [His^1]actin) and D1HD4H (replacement of both Asp-1 and Asp-4 with His; [His^1,His^4]actin), were expressed in *D. discoideum* cells. These recombinant actins were purified by a procedure that uses neither detergents nor denaturants. Biochemical properties of the purified recombinant actins were examined.

MATERIALS AND METHODS

Plasmid Construction. The plasmid pSC79 that carries the actin 15 coding sequence and its 5′ promoter and 3′ terminator sequences (4) was kindly provided by David Knecht (University of Connecticut). Mutations were introduced into the actin 15 gene by oligonucleotide-directed mutagenesis (18, 19) so that Asp-1 and/or Asp-4 of actin 15 were replaced with His. The Asp → His changes were chosen because they generated new restriction sites along the actin 15 gene that were used to select mutants and also because the changes facilitated separation of mutant actins from wild-type (wt) actin by DEAE-5PW chromatography as below.

Vectors were then constructed by inserting mutated actin 15 genes into pnDeI (20) by using procedures described by Sambrook *et al.* (21). The resulting transformation vectors used to produce D1H and D1HD4H mutant actins were designated as plasmid 105 and plasmid 114, respectively.

Transformation of *Dictyostelium* Cells. *Dictyostelium* cells (1×10^7 cells per ml) were transformed with plasmid 105 or plasmid 114 (20 μg/ml) by electroporation with a Gene Pulser (Bio-Rad) (22). After incubation of electroporated cells for 18 h in HL5 medium (23) containing penicillin/streptomycin solution (GIBCO; 6 μl/ml of medium), transformed cells were selected by growing them in the presence of an antibiotic, G418 (geneticin; 10 μg/ml) (22). The G418-resistant cells were cultured in HL5 containing G418 (10 μg/ml) and penicillin/streptomycin on a 9-cm sterile plastic dish for a week. Cells were then transferred on three larger sterile dishes (10 cm × 14 cm). When these dishes were covered by the cells, they were further transferred to an agitated 500-ml culture in the above medium.

Preparation of Actin. When the number of *Dictyostelium* cells reached 1×10^9 in a 500-ml culture, they were harvested by centrifugation at 1000 × *g*. Cells were then washed twice with 30 mM Mops, pH 7.0/2.5 mM EGTA/5 mM $MgCl_2$/0.2

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Abbreviations: D1H, [His^1]actin mutant; D1HD4H, [His^1,His^4]actin mutant; wt, wild type; DTT, dithiothreitol; HMM, heavy meromyosin; S1, myosin subfragment 1; F, filamentous.

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mM ATP and finally suspended in 15 ml of the above buffer. Then, 0.01 vol of 100 mM phenylmethylsulfonyl fluoride in dimethyl formamide, leupeptin (10 mg/ml in water), chymostatin (2 mg/ml in dimethyl sulfoxide), and pepstatin (2 mg/ml in dimethyl sulfoxide) were added. Cells were sonicated for six 10-sec periods and centrifuged at 10,000 × *g* for 10 min. The supernatant was then centrifuged at 541,000 × *g* in a TL-100 ultracentrifuge (Beckman) for 1 h. The supernatant of the high-speed centrifugation was filtered through Millex-HV (0.45 μm) (Millipore) and directly applied to a DEAE-5PW HPLC column (Tosoh, Tokyo). Elution was carried out by a linear gradient of 0–0.5 M NaCl in 20 mM Tris·HCl (pH 8.0); wt actin and a mutant actin were eluted as separate peaks. Immediately after elution of the mutant actin, 0.2 mM ATP, 0.2 mM $CaCl_2$, and 5 mM $MgCl_2$ (final concentrations) were added to the peak fraction to facilitate actin polymerization. The fraction was then dialyzed against 30 mM Mops, pH 7.0/5 mM $MgCl_2$/0.2 mM ATP/0.2 mM $CaCl_2$/0.2 mM dithiothreitol (DTT). The resulting filamentous (F)-actin was centrifuged at 541,000 × *g* for 30 min in the TL-100 centrifuge. The F-actin pellet was resuspended in 2 mM Tris·HCl, pH 8.0/0.2 mM $CaCl_2$/0.2 mM ATP/0.2 mM DTT and dialyzed against the same solvent. The resulting solution was finally cleared by centrifugation at 541,000 × *g* for 1 h in the TL-100 centrifuge. The supernatant contained the homogeneous mutant actin. The yield of the purified actin was 500 μg from 1×10^9 cells. After harvesting the *Dictyostelium* cells, all procedures were carried out on ice or at 4°C except processing through HPLC columns. The wt actin was purified by the procedure above from wt Ax2 cells.

ATPase Measurements. The wt and mutant actins were polymerized by addition of 10 mM KCl/10 mM Mops/5 mM $MgCl_2$/phallacidin (Sigma; 5 μg/ml) (final concentrations). After incubation at 25°C for 2 h, F-actin was centrifuged at 541,000 × *g* for 10 min in the TL-100 centrifuge. The soft pellet was dispersed in 2.5 mM KCl/10 mM Mops/4 mM $MgCl_2$/0.2 mM DTT/phallacidin (5 μg/ml) (ATPase buffer). It was again centrifuged at 541,000 × *g* for 10 min. The resulting pellet was dispersed again in the ATPase buffer. This procedure was repeated one more time. The resulting solution was used as F-actin. Heavy meromyosin (HMM), prepared from rabbit skeletal myosin (24), and F-actin were mixed to make a 135-μl reaction mixture containing HMM at 30 μg/ml and various concentrations of F-actin in the ATPase buffer. The ATPase reaction was initiated by addition of 0.1 vol of 10 mM ATP. After various periods at 23°C, 10 μl of the reaction mixture was drawn and the reaction was stopped by addition of 190 μl of ice-cold 0.2 M perchloric acid. Amounts of released phosphate were quantitated by the modified malachite green method (25) and were plotted against reaction time to obtain the phosphate release rate. Actin-activated ATPase activity (V) was then obtained by subtracting ATPase activity of HMM from the rates determined above. The apparent affinity of actin and ATP-saturated myosin (K_{app}) and the maximum turnover rate at infinite actin concentration (V_{max}) were obtained by a double-reciprocal plot.

Other Assays. *In vitro* motility assays (26–28) were carried out according to the method of Yano-Toyoshima *et al.* (28). Briefly, HMM (20 μg/ml) in 25 mM KCl/25 mM imidazole, pH 7.4/4 mM $MgCl_2$/1 mM DTT (assay buffer) was bound to coverslips coated with a nitrocellulose film. Then, wt and mutant actin filaments (20 nM) labeled with tetramethylrhodamine phalloidin (Molecular Probes) were loaded onto the coverslips. After addition of 1 mM ATP, translational movement of actin filaments was monitored by a fluorescence microscope (Axioplan; Zeiss) equipped with a high-sensitivity TV camera (CTC9000; Ikegami, Tokyo) and recorded by a video recorder. The assay was carried out at 22.5°C.

Binding of myosin subfragment 1 (S1) to actin filaments was measured by pelleting experiments. To 12 μg of F-actin in the ATPase buffer were added various amounts of S1 prepared from rabbit skeletal myosin (24). The final volume was adjusted to 90 μl with the ATPase buffer. After 20 min at 0°C, 10 μl of 10 mM ATP were added. Immediately after addition of ATP, the reaction mixtures were centrifuged at 4°C for 8 min at 436,000 × *g*. Pellets were resuspended in 20 μl of the ATPase buffer and used as samples for the Na$DodSO_4$ gel electrophoresis (29). Two to three independent series of pelleting experiments were carried out for each actin species. The Na$DodSO_4$ gel electrophoresis was carried out at least twice for each sample by using the PhastSystem (Pharmacia). Band intensities of actin and S1 heavy chain were measured by scanning the gels with a Beckman DU64 and the measurements were then averaged.

RESULTS

Expression of Mutant Actins in *Dictyostelium* Cells. Two-dimensional gel patterns (30) of *Dictyostelium* cells transformed with pnDeI (Ax2-pnDeI) and those with plasmid 114 (Ax2-114) revealed that Ax2-114 produced a polypeptide not present in Ax2-pnDeI, as shown by a double arrowhead in Fig. 1. We concluded that the polypeptide was the mutant actin (D1HD4H) by considering the following facts. (*i*) The polypeptide cross-reacted with an antibody raised against the N terminus of *Dictyostelium* actin (data not shown), (*ii*) its apparent molecular weight was very close to that of wt actin (single arrowheads in Fig. 1), and (*iii*) its isoelectric point was more basic than that of wt actin as expected from the Asp → His replacement. It should be noted here that, when *Dictyostelium* cells were transformed with plasmid 114, both endogenous wt actin and mutant D1HD4H actin were expressed simultaneously since the transformation vector duplicated outside the chromosome without integration (20).

To estimate the amount of D1HD4H actin expressed in *Dictyostelium* cells, whole lysate of Ax2-114 was analyzed on a one-dimensional Na$DodSO_4$ gel. Because of the slight difference in mobilities of mutant and wt actins, they were visible as separate bands (wt, arrowhead; D1HD4H, double arrowheads) (Fig. 2). Intensities of these bands showed that expression of endogenous wt actin was much less than that of D1HD4H actin; the ratio of D1HD4H actin to wt actin was almost 2:1. However, the total amount of actin in Ax2-114 (i.e., wt actin plus D1HD4H actin) was virtually equal to the amount of wt actin in Ax2-pnDeI. In other words, expression

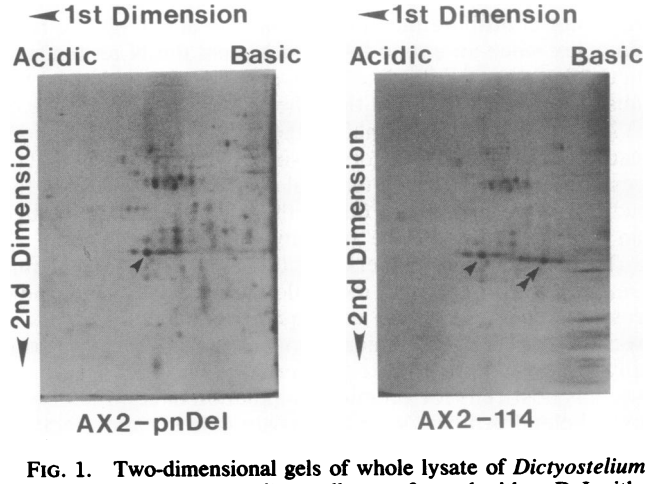

FIG. 1. Two-dimensional gels of whole lysate of *Dictyostelium* cells Ax2-pnDeI (*Dictyostelium* cells transformed with pnDeI without actin gene, expressing only endogenous wt actin) and Ax2-114 (*Dictyostelium* cells transformed with plasmid 114, expressing both endogenous wt actin and mutant D1HD4H actin). Single and double arrowheads indicate wt actin and D1HD4H actin, respectively. Gels were silver-stained.

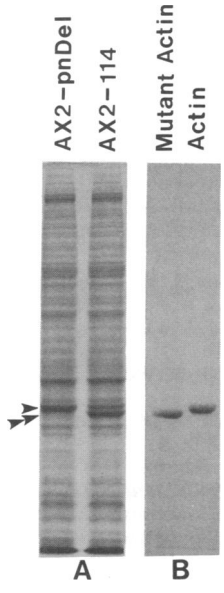

FIG. 2. (*A*) One-dimensional $NaDodSO_4$ gels of whole lysates from Ax2-pnDel and Ax2-114. Single and double arrowheads indicate positions of wt and D1HD4H actins, respectively. (*B*) Purified wt and mutant (D1HD4H) actins. Note the slight difference of mobilities of wt and mutant actins. Gels were stained with Coomassie blue.

of the mutant actin was compensated by a lower expression level of the endogenous wt actin in Ax2-114.

Actin-Activated ATPase of HMM. D1H and D1HD4H actins purified from the transformed cells formed filaments as did wt actin. In the absence of ATP, these filaments were decorated with HMM to form arrowheads (data not shown).

Actin-activated ATPase activities of HMM were measured in the presence of various amounts of wt, D1H, or D1HD4H actin filaments to assess an effect of the Asp → His changes. The apparent affinity of actin for ATP-saturated HMM (K_{app}) and the maximum turnover rate at infinite actin concentration (V_{max}) were obtained from the double-reciprocal plot shown in Fig. 3. With the first replacement Asp-1 → His, the V_{max} values dropped from 14.6 sec^{-1} to 6.4 sec^{-1} whereas the K_{app} values hardly changed (7 μM vs. 6 μM). The V_{max} value dropped further to 1.2 sec^{-1} upon the second replacement Asp-4 → His, without significant change in the K_{app} values (7 μM vs. 5 μM).

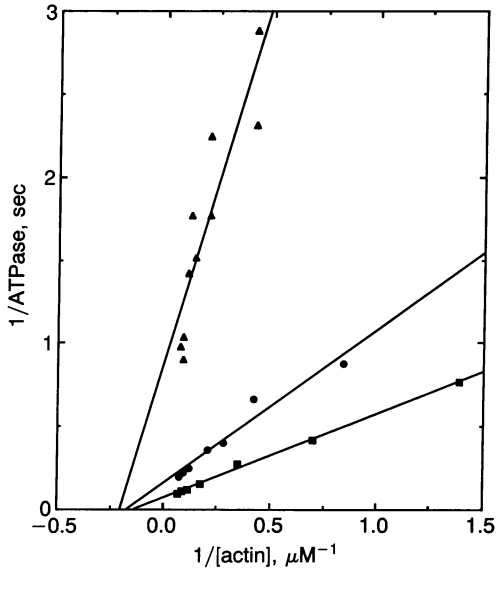

FIG. 3. Double-reciprocal plots of the actin-activated ATPase activity of HMM. ■, wt actin filaments; ●, D1H actin filaments; ▲, D1HD4H actin filaments. The ATPase activity was measured at 23°C in 2.5 mM KCl/10 mM Mops/4 mM $MgCl_2$/0.2 mM DTT/phallacidin (5 μg/ml) (ATPase buffer). HMM was at 30 μg/ml.

To confirm the above result that the mutations hardly affected the apparent affinity of actin and ATP-saturated myosin, mixtures of actin and various concentrations of S1 were centrifuged in the presence of ATP. Molar ratio values of actin and S1 that sedimented as a complex were measured. Consistent with the ATPase measurements, S1 concentrations needed to saturate half of the actin monomers hardly changed by the mutations. As shown in Fig. 4, the S1 concentration for half-saturation increased slightly from 4.5 μM to 5.5 μM with the first Asp → His replacement (■ vs. ●), whereas no further increase was observed upon the second Asp → His replacement (● vs. ▲).

Motility of wt and Mutant Actins. Sliding velocity of actin filaments driven by HMM was measured by using a fluorescence microscope. The velocity ranged from 1.0 μm/sec to 2.5 μm/sec for wt filaments and from 0.5 μm/sec to 2.0 μm/sec for D1H filaments. The velocity distribution of wt and D1H filaments shown in Fig. 5 clearly indicates that D1H filaments moved more slowly than wt filaments on the average; the average velocity was 1.95 ± 0.33 μm/sec for wt filaments and 1.34 ± 0.30 μm/sec for D1H filaments.

Behavior of D1HD4H filaments was different from the behavior of wt and D1H filaments. Virtually all wt and D1H filaments were moving when examined whereas only a part of D1HD4H filaments were moving under the same condition. D1HD4H filaments moved for a while, stopped, and then resumed the movement again. On the average, ≈20% of the D1HD4H filaments underwent translational movement at slower rates than the wt and D1H filaments. Their fastest velocity was 0.75 μm/sec. However, velocity of the majority of moving filaments was much lower, 0.25 μm/sec. The average velocity was 0.41 ± 0.13 μm/sec. Although the majority of D1HD4H filaments did not move under the assay conditions, they were potentially motile since they started to move on raising the temperature from 22.5°C to 30°C.

DISCUSSION

Chemical crosslinking experiments showed that acidic residues at the N- and C-terminal regions of actin are at the actin–myosin interface (6, 7). Functional roles of the acidic residues at the N terminus during the ATPase cycle of

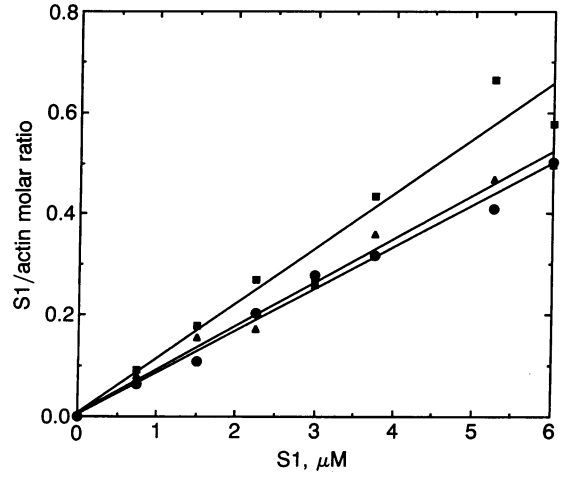

FIG. 4. Molar ratio of actin and S1 in pellets. Pelleting experiments were carried out at 4°C in the same buffer as in Fig. 3. ■, wt actin; ●, D1H actin; ▲, D1HD4H actin.

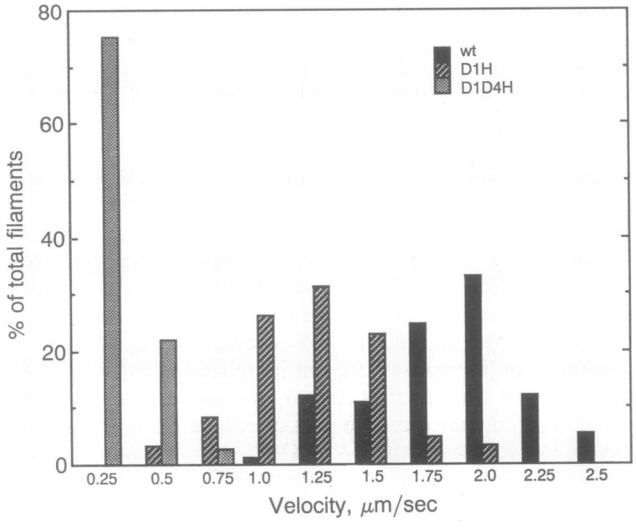

FIG. 5. Velocity distribution of translational movement of actin filaments driven by HMM. Measurements were carried out at 22.5°C in 25 mM KCl/25 mM imidazole, pH 7.4/4 mM $MgCl_2$/1 mM DTT. Note that only moving filaments were counted for D1HD4H actin.

actomyosin were assessed by anti-peptide antibodies raised against the region (13). These antibodies and their fragments inhibited the actin-activated myosin ATPase. We can interpret the result by assuming that the N terminus of actin is essential for activating the myosin ATPase activity. Another interpretation is that the ATPase activation is inhibited through an allosteric effect of the bound antibody even though the N terminus is not functionally involved in the activation process of the myosin ATPase reaction.

The present experiments showed that the disruption of the acidic residue cluster resulted in loss of activation of the myosin ATPase reaction and also loss of the myosin-driven sliding movement of actin filaments. The results strongly support the hypothesis that ionic interactions of the N-terminal acidic residues of actin with myosin are important for the ATP-dependent actin–myosin interaction, which is the essential process for the sliding movement of actin and myosin filaments and thus for the force generation.

The Asp → His replacements diminished the actin-activated ATPase activity of myosin by affecting the V_{max} value without an appreciable change in the K_{app} value. In contrast to the Asp → His replacements, subtilisin cleavage of actin at Met-47 and Gly-48 affected the K_{app} value, not the V_{max} value (31). Thus, it is possible to modulate the apparent actin–myosin affinity K_{app} and the maximum turnover rate V_{max} of the myosin ATPase reaction independently by altering particular structures of the actin molecule. One can speculate that these actin structures correspond to the part of the functional subdomains that participate in binding the actin–myosin complex or in activating the myosin ATPase during the actomyosin ATPase cycle.

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