Actin-interacting protein 1 (AIP1) is a WD40 repeat protein that enhances actin filament disassembly in the presence of actin-depolymerizing factor (ADF)/cofilin. AIP1 also caps the barbed end of ADF/cofilin-bound actin filament. However, the mechanism by which AIP1 interacts with ADF/cofilin and actin is not clearly understood. We determined the crystal structure of Caenorhabditis elegans AIP1 (UNC-78), which revealed 14 WD40 modules arranged in two seven-bladed β-propeller domains. The structure allowed for the mapping of conserved surface residues, and mutagenesis studies identified five residues that affected the ADF/cofilin-dependent actin filament disassembly activity. Mutations of these residues, which reside in blades 3 and 4 in the N-terminal propeller domain, had significant effects on the disassembly activity but did not alter the barbed end capping activity. These data support a model in which this conserved surface of AIP1 plays a direct role in enhancing fragmentation/dissociation of ADF/cofilin-bound actin filaments but not in barbed end capping.

Actin filaments are the core components of the highly dynamic actin cytoskeleton, which plays an essential role in a wide range of biological processes. Regulated fragmentation and depolymerization of actin filaments are important for disassembling specific cytoskeletal structures and maintaining high concentrations of monomeric actin, thereby enhancing actin filament turnover. Actin-depolymerizing factor (ADF)/cofilin plays a central role in the disassembly process by accelerating monomer dissociation from the pointed ends and severing filaments (reviewed in Refs. 1–4).

Actin-interacting protein 1 (AIP1) is a unique regulator of ADF/cofilin-mediated actin dynamics (reviewed in Ref. 5). The interaction between AIP1 and actin was originally detected by a yeast two-hybrid system (6). In vitro, AIP1 alone has negligible or very weak effects on actin dynamics, whereas, in the presence of ADF/cofilin, AIP1 enhances filament fragmentation (7–9). In addition, AIP1 has the ability to cap barbed ends and bind to the side of ADF/cofilin-bound filaments (10). Although the capping activity has been suggested to enhance filament fragmentation by preventing reannealing of the severed filaments (10, 11), direct microscopic observation of the effects of AIP1 on ADF/cofilin-bound filaments indicates that AIP1 actively disassembles ADF/cofilin-bound filaments, and simple barbed end capping by other capping agents does not enhance disassembly by ADF/cofilin (12). At present, the biological significance of the capping activity of AIP1 is not well understood.

The mechanism by which AIP1 selectively interacts with ADF/cofilin-bound actin filaments is unknown. Actin residues in subdomains 3 and 4 participate in the two-hybrid interaction with yeast AIP1 (6). The AIP1-interacting residues in subdomain 3 are also required for interaction with cofilin (9), suggesting that AIP1 directly interacts with subdomain 4 of actin and indirectly with subdomain 3, which might be mediated by endogenously expressed yeast cofilin. The C terminus of ADF/cofilin is required for binding to F-actin, but not to G-actin (13, 14), and also for the two-hybrid interaction with AIP1 (9) and activation of filament disassembly by AIP1 (15). ADF/cofilin interacts with two actin subunits in the filament (16), and the C terminus of ADF/cofilin is predicted to be close to subdomains 2 and 1 of the lower actin subunit in the filament (14). Therefore, this region of ADF/cofilin is not likely to be a binding site for AIP1. Rather, the C terminus of ADF/cofilin might participate in modulating the twist of the filament (16, 17) or stabilizing a particular conformation of the filament (18, 19), which might support binding of AIP1.

AIP1 is conserved among eukaryotes and is involved in the cytoskeletal regulation in vitro. In budding yeast, an AIP1-null mutant is viable but synthetic lethal in combination with cofilin alleles (9, 20). Yeast AIP1 co-localizes with cofilin to the cortical actin patches, and an AIP1 null mutation results in...
mislocalization of cofillin to cable-like structures (9, 20). In Caenorhabditis elegans, mutations of the unc-78 gene, which encodes AIP1, cause disorganization of actin filaments in the body wall muscle (21), and unc-78 shows genetic interactions with the unc-60B ADF/cofilin gene that is required for proper organization of muscle actin filaments (22). UNC-78/AIP1 strongly disassembles UNC-60B-bound actin filaments but shows very weak effects on actin filaments in the presence of the ubiquitously expressed UNC-60A (15, 23, 24). In Dictyostelium discoideum, an AIP1-null mutation partially impairs several actin-dependent cellular processes, including cytokinesis and cell motility (25, 26). In Arabidopsis, AIP1 co-localizes with ADF (27) and plays essential roles in actin organization and plant development (28). In cultured Drosophila cells, cofillin (Twinstar) and AIP1 are required for lamella formation (29). Co-localization of AIP1 with ADF/cofilin is also reported in other organisms (reviewed in Ref. 5), suggesting that they are evolutionarily conserved co-regulators for actin cytoskeleton.

We determined the crystal structure of C. elegans UNC-78/AIP1 at 1.9-Å resolution, which allowed for the identification of highly conserved surface residues. Mutagenesis studies targeted 20 of these residues and identified five residues that alter the activity of UNC-78/AIP1. Importantly, these mutations uncouple filament disassembly activity from capping activity. Our results also highlight the location of a functionally relevant surface of UNC-78/AIP1 and suggest distinct roles for filament binding and disassembly.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Rabbit skeletal muscle actin was purified as described (30). Bacterially expressed recombinant UNC-60B was purified as described previously (23). Bacterially expressed recombinant UNC-78 with no tag was purified as described previously (15). Recombinant glutathione S-transferase (GST) was expressed from pGEX-2T (Amersham Biosciences) in Escherichia coli BL21 (DE3) and purified with glutathione-Uniflow (BD Biosciences Clontech) following the manufacturer’s instruction.

**Crystallization and Data Collection**—Recombinant untagged UNC-78 protein was dialyzed against 20 mM Tris-Cl, 50 mM KCl, 1 mM dithiothreitol, pH 7.5, and concentrated to 3.5-7.0 mg/ml for crystallization. Initial crystallization conditions were determined by hanging drop vapor diffusion using equal volumes of the protein (3-4 mg/ml) and a precipitant suspended above 1 ml of the precipitant. Clusters of plates grew from 0.1 M MES, 20% polyethylene glycol 8000, 10 mM MnCl₂, 5% glycerol, pH 6.0. After careful desiccation, a small fragment was transferred to mother liquor supplemented with 20% glycerol as a cryoprotectant and flash-cooled to 100 K prior to data collection. Diffraction was consistent with the monoclinic space group P2₁, (unit cell dimensions: \( a = 40.66, b = 90.82, c = 76.98 \) Å, \( \beta = 94.06^\circ \)), and native data were collected to 2.0 Å using a MARCCD and radiation of \( \lambda = 0.98 \) Å at National Synchrotron Light Source beamline X9A. All attempts to obtain heavy atom derivatives for these crystals were unsuccessful, as were efforts to produce selenomethionyl-derivatized protein.

The addition of 2.44 mM n-octanolsuccrose resulted in the growth of small single crystals, which were used for microseeding. Single crystals grew to a size of 0.3 × 0.4 × 0.25 mm³ after 2 weeks at 18 °C. Diffraction from these crystals extended to 1.6-Å resolution and was consistent with the monoclinic space group P2₁, (unit cell dimensions: \( a = 64.85, b = 65.174, c = 69.24 \) Å, \( \alpha = \beta = 90^\circ, \gamma = 98.27^\circ \)). Data from native and derivative crystals were collected at National Synchrotron Light Source beamline X9B using an ADSC Quantum 4 CCD detector at a temperature of 100 K, processed with the HKL 2000 package, and scaled and merged with SCALPACK (31) (see Table I for a summary of data collection). Native data were collected to 1.70-Å resolution, using 0.98-Å wavelength radiation. A high quality derivative was prepared by soaking crystals in mother liquor containing 2 mM sodium salt of ethylenecarbolllyclic acid for 5 h prior to freezing. Single anomalous dispersion data were collected to 1.6 Å using a wavelength of 1.0075 Å.

**Structure Determination and Refinement**—The heavy atom constellation was solved with SHELXD, which identified four mercury sites, and phases were calculated with SHELX (32). The resulting map was of excellent quality and allowed for automated model building with ARPWiARP (33), which fit 574 of 611 residues, with an \( R_{	ext{ cryst }} \) and \( R_{	ext{free }} \) of 32.4 and 35.8%, respectively. The model of the mercury-derivatized UNC-78 was completed with alternating rounds of refinement with CNS (34) and manual rebuilding with O (35), which resulted in \( R_{	ext{ cryst }} \) and \( R_{	ext{free }} \) of 28.0 and 30.2%, respectively. AMORE (36) was used to orient the structure of the mercury derivative in the native unit cell. The native structure was refined with maximum likelihood simulated annealing, as implemented in CNS. Solvent molecules were built into 3.5 Å peaks in difference Fourier syntheses, and a Mn⁺² ion was subsequently assigned on the basis of observed electron density, coordination number, geometry, and refined temperature factors. The final model (1NR0) consists of 4581 protein atoms, a single Mn⁺², and 716 water molecules with converged \( R_{	ext{ cryst }} \) and \( R_{	ext{free }} \) of 19.9 and 23.0%, respectively, using all data between 23.6 and 1.7 Å (Table I and Fig. 1E).

The structure of the first crystal form was solved by molecular replacement with CNS, using 1NR0 structure as the search model. The final model (1PEV) consisted of 4600 protein atoms and 330 water molecules with converged \( R_{	ext{ cryst }} \) and \( R_{	ext{free }} \) of 19.7 and 23.4%, respectively, using all data between 21.7- and 2.0-Å resolution. No Mn⁺² was detected in the structure (Table I).

**Expression and Purification of Recombinant GST-UNC-78 Protein**—The full-length UNC-78 cDNA was digested with BamHI and HindIII from the pET-UNC-78 vector (15) and cloned into pGEX-2T (Amersham Biosciences) between BamHI and SmaI sites (pGEX-UNC-78). The E. coli strain BL21 (DE3) was transformed with pGEX-UNC-78 and cultured in M9ZB medium containing 50 μg/ml ampicillin at 37 °C until \( A_{600} \) reached 0.6 cm⁻¹. Then the culture was cooled to room temperature, and protein expression was induced by adding 0.1 mM isopropyl β-D-thiogalactopyranoside for 3 h at room temperature. The cells were harvested by centrifugation at 5000 × g for 10 min and disrupted by a French pressure cell at 5000–8000 pounds/in² in phosphate-buffered saline containing 0.2 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 20,000 × g, and the supernatants were applied to a glutathione-Uniflow column (BD Bio-

### Table I: Summary of diffraction data

| Data reduction | Native | Native | EMTS |
|----------------|--------|--------|------|
| PDB entry      | 1PEV   | 1NR0   |      |
| Space group    | P2₁    | P2₁    | P2₁ |
| Unit cell      | \( a = 40.66, b = 90.82, c = 76.98 \) Å, \( \alpha = \beta = 90^\circ, \gamma = 94.06^\circ \) | \( a = 64.85, b = 65.174, c = 69.24 \) Å, \( \alpha = \beta = 90^\circ, \gamma = 98.27^\circ \) | \( a = \beta = 90^\circ, \gamma = 98.28^\circ \) |
| Resolution (Å) | 21.7 to 2.0 | 23.6 to 1.7 | 23 to 1.60 |
| Completeness of data set (%) | 97.0 | 98.6 | 95.9 |
| Average \( I / \sigma I \) of data set | 16.1 | 19.2 | 18.9 |
| Completeness of outermost shell (%) | 87.8 | 97.7 | 84.1 |
| Average \( I / \sigma I \) of outermost shell | 3.4 | 5.0 | 4.1 |
| \( R_{	ext{free}} \) (I) (%) | 5.8 | 4.5 | 4.2 |
| Phasing power | 1.23 |
| Overall figure of merit | 0.43 |

\( R_{\text{free}} = \sum I_{h} - \sum F_{h} / \sum F_{h} \), where \( I \) is the mean intensity of the \( h \) observations of reflection \( h \).

\( I \) Phasing power = r.m.s. ((\( F_{h} / E \)), where \( F_{h} \) is the calculated structure factor of the heavy atoms, and \( E \) is the residual lack of closure.


**Table II**

| Mutagenesis primers | Sequence |
|---------------------|----------|
| Y73A-S | CGAACATTGAGCAAGGGAGATGAG |
| K85A-S | CGGAGAAGACTGCGATAGG |
| K96A-S | CGGAGAAGACTGCGATAGG |
| E114A-S | TGCACCTATTGAGGCAAGGGAGATGAG |
| D123A-S | TGCACCTATTGAGGCAAGGGAGATGAG |
| D125A-S | TGCACCTATTGAGGCAAGGGAGATGAG |
| E123A-S | TGCACCTATTGAGGCAAGGGAGATGAG |
| E114A-S | TGCACCTATTGAGGCAAGGGAGATGAG |
| D135A-S | TGCACCTATTGAGGCAAGGGAGATGAG |
| F128A-S | TGCACCTATTGAGGCAAGGGAGATGAG |
| R125A-S | TGCACCTATTGAGGCAAGGGAGATGAG |
| E123A-S | TGCACCTATTGAGGCAAGGGAGATGAG |
| E114A-S | TGCACCTATTGAGGCAAGGGAGATGAG |
| K96A-S | TGCACCTATTGAGGCAAGGGAGATGAG |
| Y73A-S | TGCACCTATTGAGGCAAGGGAGATGAG |

**RESULTS**

Structure of UNC-78—The UNC-78 structure revealed two distinct domains that pack to give an overall appearance of a distorted “figure 8” (Fig. 1A). This organization results in “conceal” and “convex” surfaces, which may be relevant to function (see discussion below). Each individual domain is composed of a seven-bladed β-propeller first described for the β-subunit of the heterotrimERIC G-protein transducin (38). Similar to other seven-bladed propellers, the UNC-78 domains are characterized by a diameter and height of ~50 and ~30 Å, respectively. The two domains exhibit significant structural similarity and can be superimposed with a 2.5 Å r.m.s. deviation for 274 Cα pairs. By convention, the individual blades are numbered from 1 to 14, starting with the N terminus (Fig. 1A). Each blade is composed of a four-stranded antiparallel β-sheet, with the individual strands labeled A to D starting from the center of the propeller (Fig. 1A, middle). The individual sheets exhibit significant twist, such that the A strands run approximately parallel to the propeller axis and form the walls of a solvent-filled central channel (~12–13 Å in diameter). The strands become progressively more tilted as they radiate from the center of the molecule, with the D strands running approximately perpendicular to the propeller axis. The N-terminal propeller is formed by a continuous chain segment, residues 13–323, which defines blades 1–7, whereas the C-terminal propeller is discontinuous, with two chain segments, residues 1–12 and 324–611, forming blades 8–14. The angular displacement between neighboring blades around the pseudo-7-fold axis deviates from ideality (i.e. 51.4°) varying from 42 to 62° for individual pairs of blades. The 14 blades can be superimposed with r.m.s. deviations of ~2.0 Å for ~40 Cα pairs, with the significant structural differences predominately confined to the loops that join the individual β-strands (data not shown).

As first observed in the β-subunit transducin (38), the first six blades are formed by a continuous polypeptide segment that contributes strands A–D. Closure of these domains is typically achieved by the utilization of the immediate N terminus as the outer (D) strand for the seventh blade. Prior to the correct structure, the only known exception was prolyl oligopeptidase, in which all seven blades are formed by continuous segments, and domain closure is accomplished by considerable hydrophobic interactions between the first and seventh blades (39). Notably, both propeller domains in UNC-78 lack the generic mode of domain closure (Fig. 1A, middle and right). Reminiscent of the prolyl oligopeptidase, the N-terminal domain of UNC-78 is stabilized by hydrophobic interactions involving hydrophobic residues from blade 1 (Val22 Val23, Leu24 Ile33 and Val46) and blade 7 (Ile291 Val296, Ile300 and Ile306). The C-terminal propeller in UNC-78 utilizes a variant of the typical closure mechanism, in which the immediate N terminus of the protein stretches across the interdomain interface to form the outer (D) strand of blade 14 (Fig. 1A, right).

The distorted “figure 8” organization is the consequence of specific packing interactions along the circumference of the propeller domains. These distortions are manifested in the relative rotations of the two domains around all three axes passing though the center of the molecule (Fig. 1A, left), resulting in a pseudo-2-fold relationship between the domains. The interface is stabilized by several main chain-main chain hydrogen bonds, including Leu19 N-Thr20 O, Leu19 N-His325 O, Arg15 N-Cys326 O, Ala1263 O-Ala244 N, and Ile237 N-Gly259 O (Fig. 1B).
**FIG. 1. Structure of UNC-78.** A, ribbon diagram of UNC-78 showing two covalently linked seven-bladed $\beta$-propellers. The nomenclature used to describe the blades and strands is shown. **Middle**, view down the axis of the N-terminal $\beta$-propeller domain; **left**, side view of UNC-78 showing the concave and convex surfaces. This orientation is obtained by a 90° rotation about the vertical axis relative to the **middle image**. The arrow identifies the approximate location of the pseudo-2-fold axis (i.e. $\sim 167^\circ$) that relates the two individual domains. **Right**, view down the axis of the C-terminal $\beta$-propeller domain. This orientation is obtained by successive rotations of 60 and 20° about the horizontal and vertical axes relative to the **middle image**. B and C, the hydrogen bonds that are important for the domain/domain interface in UNC-78. B, five selected main chain-main chain hydrogen bonds are marked: Lys9 N-Thr50 O(a), Ile327 N-Gly599 O(b), Ala326 O-Ala344 N(c), Leu13 N-His323 O(d), and Arg15 N-Cys36 O(e). C, side chain-side chain hydrogen bonds between conserved residue His323 of the first domain and Ser341 and Asp343 of the second domain are shown. The side chain of conserved residue Trp351 stabilizes this interaction. D, superposition of the C. elegans (red) and S. cerevisiae (blue) AIP1 (PDB code 1PI6). The two molecules were superimposed on the basis of the N-terminal $\beta$-propeller domains, which highlights the $\sim 9^\circ$ greater
The side chains of the conserved residues His\textsuperscript{322}, Ser\textsuperscript{341}, and Trp\textsuperscript{357} participate in hydrogen bonds between blades 7 and 8 to further stabilize the interface (Fig. 1C). As the consequence of the two connecting polypeptide segments, there are a number of conserved residues contributed from blades 1, 7, 8, and 14 that are located in proximity to the interface, although they do not make direct contacts. These residues include Tyr\textsuperscript{35} and Gly\textsuperscript{394} of blade 1; Gly\textsuperscript{304}, Gly\textsuperscript{322}, His\textsuperscript{323}, Lys\textsuperscript{325}, and His\textsuperscript{327} of blade 7; Gly\textsuperscript{346} and Trp\textsuperscript{351} of blade 8, and Asp\textsuperscript{601} and Pro\textsuperscript{14} of blade 14. The validity of this interface is further supported by the observation of a nearly identical organization in the two independent structures of UNC-78 (1NR0 and 1PEV; Table I), which superimpose with an r.m.s. deviation of 0.8 \AA over all 610 C\textsubscript{\textalpha} pairs. Superposition of the individual domains reveals a slight \textdegree rotation between the two domains in the crystal forms, which might be the consequence of packing interactions.

Subsequent to the deposition of the UNC-78 structures to the Protein Data Bank, the structure of the homologous Saccharomyces cerevisiae Aip1 structure was reported (40). The C. elegans and S. cerevisiae share 30% sequence identity and exhibit nearly identical topologies, with an r.m.s. deviation of 2.0 \AA over 573 C\textsubscript{\textalpha} pairs. Superposition of individual domains demonstrates subtle differences in overall organization, in which the two domains of the yeast protein are rotated \textdegree towards each other, resulting in a slightly more closed arrangement than observed in the C. elegans protein (Fig. 1D). The independent structure of the yeast protein supports a widely conserved overall structural organization and further suggests some interdomain variability, which might be important for function.

Activity of GST-UNC-78—We previously reported purification of bacterially expressed UNC-78 without a fusion tag sequence (15). We found that UNC-78 could be expressed in E. coli as a soluble fusion protein with GST and rapidly purified by glutathione affinity chromatography. Although a thrombin recognition sequence was present at the junction between GST and UNC-78, thrombin failed to cleave GST from UNC-78 (data not shown). GST-UNC-78 disassembled F-actin only in the presence of UNC-60B (Fig. 2A, compare the amounts of actin in the supernatants in lanes 1 and 7) and in the same manner as untagged UNC-78 (Fig. 2A, compare lanes 1 and 7). GST alone did not interact with F-actin in the absence (Fig. 2A, lanes 5 and 6) or the presence of UNC-60B (Fig. 2A, lanes 11 and 12). Quantitative analysis of the pelleting assays indicated that GST-UNC-78 disassembled UNC-60B-bound F-actin to the same extent as untagged UNC-78 (Fig. 2B). These results strongly suggest that GST-UNC-78 has indistinguishable activity from UNC-78 with no tag. Therefore, we used GST-UNC-78 as a template of the following mutagenesis study and tested the activity of mutants as GST fusion proteins.

Identification of Conserved Surface Residues of UNC-78—Based on functional conservation among the AIP1 proteins from different species, we reasoned that conserved solvent-accessible residues were likely to play a direct role in AIP1 function. Alignment of thirteen AIP1 sequences using the Clustal method (Supplemental Fig. 1), in conjunction with solvent accessibility calculated from the UNC-78 crystal structure, allowed for the identification of the desired class of residues. As pointed out by Voegtl\textsc{i} et al. (40), we also found that the N-terminal propeller contains more conserved residues than the C-terminal propeller (Fig. 3 and Supplemental Fig. 1). Site-directed mutagenesis was performed on 20 conserved residues with high fractional accessible area, which indicates significant solvent accessibility (Fig. 3, indicated in red) (Table III). We avoided selecting many of the residues in the WD40 repeat consensus sequence. For example, aspartic acid residues between strands B and C are highly conserved among WD40 repeats (41) and probably contribute to stability and proper folding of a propeller (42).

Activity of Mutant UNC-78 Proteins to Disassemble UNC-60B-F-actin—The 20 selected residues were altered to alanine and the resultant mutant proteins examined for their ability to disassemble UNC-60B-bound actin filaments (Fig. 4). A pelleting assay was employed, in which disassembly of F-actin (10 M) was examined by a pelleting assay in the absence (lanes 1–6) or presence (lanes 7–12) of UNC-60B (20 M). The mixtures were ultracentrifuged, and the supernatants (s) and pellets (p) were analyzed by SDS-polyacrylamide gel electrophoresis. Molecular mass markers in kDa are indicated on the left. B, quantitative analysis of the pelleting assay of F-actin (10 M) with various concentrations of UNC-78 with no tag (closed symbols) or GST-UNC-78 (open symbols) in the absence (circles) or presence (triangles) of UNC-60B (20 M). Percentages of actin in the pellets were plotted as a function of the UNC-78 concentrations. Data are means \pm S.D. of three experiments.
F182A, and F192A) showed significantly decreased activity (Fig. 4, f, k, m, and o, compare open and closed symbols). In particular, E126A and D168A caused severe defects in disassembly of F-actin in the presence of UNC-60B (Fig. 4, f and k).

Remarkably, one mutant, K181A, had higher activity than wild type (Fig. 4, l). At 5 \( \mu \text{M} \) UNC-78 (K181A), nearly complete disassembly of F-actin was induced when UNC-60B was present at 1:1 molar ratio with actin (Fig. 4, l, open squares and diamonds), whereas wild type UNC-78 disassembled 60% of F-actin at maximum under the conditions examined. All other mutants showed activities that were indistinguishable from wild type (Fig 4, a–e, g–j, n, and p–t).

**Thermodynamic Stability of the Mutant UNC-78 Proteins**—Although we selected surface residues for mutagenesis, some of the mutations may alter the stability of the protein and thus indirectly affect activity. The thermodynamic stability of wild type and mutant UNC-78 proteins was examined by chemical denaturation studies utilizing urea (Fig. 5). Wild type UNC-78 with (Fig. 5a) or without GST (Fig. 5b) showed very similar denaturation patterns with decreased fluorescence intensity for the denatured states. 50% denaturation of GST-UNC-78 or untagged UNC-78 occurred at 3 \( \mu \text{M} \) urea. GST alone showed increased fluorescence at denatured states (Fig. 5c), but the fluorescence intensity was much weaker than that of UNC-78.

| Mutation | Conservation | ASAa | Actin disassembly | \( \mu \text{M} \text{ urea}^b \) |
|----------|--------------|------|-------------------|-------------------------------|
| Wild type |              |      | ++                | 2.9                           |
| F182A    | Phe (13/13)  | 0.37 | +                 | 3.0                           |
| K191A    | Lys/Arg (10/13), Ser (1/13), Gln (2/13) | 0.68 | ++                | 3.0                           |
| D250A    | Asp (13/13)  | 0.72 | +                 | 3.1                           |
| D469A    | Asp/Glu (13/13) | 0.36 | ++                | 3.2                           |
| K325A    | Lys/Arg (13/13) | 0.39 | ++                | 3.0                           |
| E345A    | Asp/Glu (13/13) | 0.47 | ++                | 3.0                           |
| D469A    | Asp/Glu (13/13) | 0.36 | ++                | 3.2                           |
| K538A    | Lys/Arg (13/13) | 0.39 | ++                | 3.0                           |

a Accessible surface area.

b Urea concentration that is required for 50% denaturation of the protein.

**Fig. 3.** Conserved surface residues of AIP1. Conserved surface residues of AIP1 that were selected for mutagenesis are shown in red. Green residues are conserved but corresponding to the consensus sequence of WD40 repeats. Blue residues are charged and highly conserved but buried inside the molecule. The structures on the left are views from the top of propellers, and those on the right are from the bottom of propeller 1. The structures are shown in space-filling models (top) and ribbon diagrams (bottom).
(Fig. 5, a and b), indicating that the GST moiety does not significantly contribute to the fluorescence of the GST-UNC-78 fusion protein.

Wild type and all mutant proteins followed a two-state unfolding transition (Fig. 5); however, there was some variability in the urea concentration required for 50% denaturation ($C_m$) for mutant UNC-78 proteins (Fig. 5 and Table III). Three mutations (E126A, D168A, and F182A) with reduced disassembly activity exhibited a decreased $C_m$ ($-2 \mu$M) (Fig. 5, i, n, and p), whereas F192A, which had a similar decrease in disassembly activity exhibited an increased $C_m$ ($-4 \mu$M) (Fig. 5r). In contrast, K181A, a hyperactive mutation, had a $C_m$ similar to wild type (Fig. 5o). A decrease in $C_m$ to $-2 \mu$M was also observed for Y73A (Fig. 5d) and K96A (Fig. 5e) without an accompanying effect on activity (Fig. 4, a and b). On the other hand, an increase in $C_m$ to $-4 \mu$M was detected for E123A (Fig. 5g) without affecting activity (Fig. 4d). Thus, whereas some of the mutant UNC-78 proteins with reduced activity had modestly altered thermodynamic stabilities, these alterations were within the range that supports properly folded and functionally active protein.

**Enhanced F-actin Binding Activity of the E126A Mutant**—Wild type UNC-78 co-sediments with F-actin in the pelleting assay (15), which is likely to represent binding to the side of the filaments as demonstrated for Xenopus AIP1 (10). By quantifying the amounts of wild type and mutant GST-UNC-78 that co-sedimented with actin, we found that E126A had enhanced activity to co-sediment with UNC-60B-bound F-actin, whereas the mutations D168A, K181A, F182A, and F192A did not change or slightly reduced this binding activity (Fig. 6). D168A, F182A, and F192A had much weaker filament disassembling activity than wild type (Fig. 4, k, m, and o) but still showed significant co-sedimentation with F-actin in the presence of UNC-60B (Fig. 6A, lanes 3–8). Molar ratios of mutant GST-UNC-78 proteins and actin were greater in the presence of UNC-60B than in the absence of UNC-60B (Fig. 6, D, F, and G, compare open triangles with closed triangles). D168A and F182A bound nearly as well as wild type (Fig. 6, D and F), but F192A exhibited weaker binding activity (Fig. 6F). K181A had enhanced filament disassembly activity (Fig. 4l). Although the majority of F-actin was disassembled, small amounts of K181A co-sedimented with residual F-actin (Fig. 6A, lanes 9 and 10), and the sedimentation was enhanced in the presence of UNC-
Fig. 5. Effects of mutations of UNC-78 on thermodynamic stability. Fluorescence intensity (arbitrary units (a.u.)) of GST-UNC-78 (a), untagged UNC-78 (b), GST alone (c), or GST-UNC-78 with mutations that are indicated on the graph (d–w) was measured after denaturation with various concentrations of urea. Data are means ± S.D. of three experiments.

60B (Fig. 6E, compare open triangles with closed triangles). Nonetheless, the activity of K181A was weaker than wild type. Interestingly, E126A showed enhanced co-sedimentation with UNC-60B and F-actin without significantly depolymerizing the filaments (Fig. 6B). The E126A mutant did not sediment in the absence of actin (Fig. 6B, lanes 1–4, 12, 15, and 16). In the absence of UNC-60B, E126A weakly co-sedimented with F-actin to similar extents observed for wild type (Fig. 6, B, lanes 1–4, and C; compare closed triangles and closed circles). However, in the presence of both actin and UNC-60B, significant amounts of E126A sedimented without increasing the amount of actin in the supernatant (Fig. 6B, lanes 7 and 8). Wild type UNC-78 co-sedimented with UNC-60B-F-actin in a similar manner except that it induced disassembly of the filament (Fig. 6B, lanes 5 and 6). Quantitative analysis of the co-sedimentation of wild type or E126A with F-actin indicated
that E126A co-sedimented with actin in a saturable manner in the presence of UNC-60B (Fig. 6C, open triangles), but co-sedimentation of wild type with actin did not reach a saturation within a range we examined (Fig. 6C, open circles). Co-sedimentation of wild type or E126A with actin in the absence of UNC-60B was not very great (Fig. 6C, closed triangles and circles). These results suggest that the E126A mutant has enhanced activity to bind to the side of UNC-60B-decorated F-actin without depolymerizing it. An equilibrium dissociation constant (K_d) for binding of E126A with UNC-60B-F-actin was 1.0 ± 0.25 μM with a saturation of 0.51 ± 0.10 mol of E126A/mol of actin. If we assume that wild type UNC-78 bound to UNC-60B-F-actin at the same stoichiometry as E126A, an estimated dissociation constant is >8 μM.

**Barbed End Capping Activity of the Mutant UNC-78 Proteins—UNC-60B severs filaments without capping ends and enhances the apparent rate of polymerization from F-actin seeds ~5-fold (Supplemental Fig. 2). In the presence of UNC-60B, wild type UNC-78, with or without a GST tag, reduced the rate of actin elongation from the F-actin seeds to similar extents, whereas UNC-78 showed no effects on the elongation in the absence of UNC-60B (Supplemental Fig. 2). These observations indicate that GST-UNC-78 caps the barbed ends in a similar manner to untagged UNC-78. Interestingly, the five mutations that altered the filament disassembly activity of UNC-78 did not affect barbed end capping activity (Supplemental Fig. 2). These mutants, E126A, D168A, K181A, F182A, and F192A, similarly reduced the rate of nucleated actin polymerization in the presence of UNC-60B and had no effects in the absence of UNC-60B (Supplemental Fig. 2). Together, these results suggest that UNC-60B-dependent capping activity was not affected by these mutations and that capping is a function of UNC-78 that is distinct and separable from filament disassembly.

**DISCUSSION**

In this study, we determined the crystal structure of UNC-78/AIP1 and identified five residues of UNC-78 that are involved in the disassembly of UNC-60B (ADF/cofilin)-bound actin filaments. These residues have distinct roles in filament disassembly but not in barbed end capping. Previously, Okada et al. (10) proposed that AIP1 enhances fragmentation by cap-

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**Fig. 6. Filamentous actin binding activity of mutant UNC-78 proteins.** A, co-pelleting assays of F-actin (10 μM) and UNC-60B (20 μM) with wild type GST-UNC-78 (2 μM) (lanes 1 and 2) or GST-UNC-78 (2 μM) with D168A (lanes 3 and 4), F182A (lanes 5 and 6), F192A (lanes 7 and 8), or K181A (lanes 9 and 10). The mixtures were ultracentrifuged, and the supernatants (s) and pellets (p) were analyzed by SDS-polyacrylamide gel electrophoresis. Molecular mass markers in kDa are indicated on the left. B, sedimentation of wild type GST-UNC-78 (5 μM) (lanes 1, 2, 5, 6, 9, 10, 13, and 14) or GST-UNC-78 (E126A) (5 μM) (lanes 3, 4, 7, 8, 11, 12, 15, and 16) was examined in the presence (lanes 1–8) or absence of F-actin (10 μM) (lanes 9–16), and in the absence (lanes 1–4 and 9–12) or presence of UNC-60B (20 μM) (lanes 5–8 and 13–16). The mixtures were ultracentrifuged, and the supernatants (s) and pellets (p) were analyzed by SDS-polyacrylamide gel electrophoresis. Molecular mass markers in kDa are indicated on the left. C–G, quantitative analysis of the co-sedimentation of F-actin with wild type GST-UNC-78 (open symbols) or mutant GST-UNC-78 (open symbols) in the absence (circles) or presence of UNC-60B (20 μM) (triangles). Results on E126A (C), D168A (D), K181A (E), F182A (F), and F192A (G) are shown. The molar ratios of GST-UNC-78 and actin in the pellets are plotted as a function of concentrations of GST-UNC-78 in the supernatants.

**Fig. 7. Locations of the functional residues of UNC-78 on the three-dimensional structure.** Mutations at Asp^{168}, Phe^{182}, or Phe^{192} (green) resulted in simple reduction in filament disassembly activity, whereas a mutation at Lys^{181} (blue) enhanced the activity. A mutation at Glu^{126} (red) reduced the disassembling activity but enhanced filament binding. Mutation sites that were found in unc-78 mutants (21) are shown in purple.
ping barbed ends and preventing reannealing of ADF/cofilin-severed filaments. However, our mutational analysis on UNC-78 demonstrates that the filament disassembly activity can be uncoupled from capping activity by point mutations, suggesting that simple capping by AIP1 is not sufficient for enhancing fragmentation/denaturation. These studies are consistent with our direct observation with fluorescence microscopy that UNC-78 strongly enhances UNC-60B-induced filament disassembly, but gelsolin or cytochalasin D does not (12), supporting an active role for UNC-78/AIP1 in filament disassembly. Although the current study does not exclude the possibility that capping is necessary for filament disassembly activity, since we have not found a mutation that abolishes capping without changing the disassembly activity. Therefore, functional relationship between capping and filament disassembly needs to be investigated by additional mutational studies.

The five residues that are important for the activity of UNC-78 map together to the concave surface of propeller 1 (Fig. 7). Asp168, Lys181, Phe182, and Phe192 are in close proximity in blade 4 and the loop connecting blades 4 and 5. Interestingly, the mutations of Asp168, Phe182, or Phe192 reduced the activity, whereas the mutation of Lys181 enhanced the activity. Therefore, this region might be a binding site for the UNC-60B-F-actin complex, but a positively charged side chain of Lys181 might have an inhibitory effect on the interaction. Glu126, which is crucial for filament disassembly but not F-actin binding, is spatially close to the other four residues but resides in blade 3 (Fig. 7). The ability of the E126A mutant to bind to UNC-60B-bound F-actin suggests that this mutation did not disrupt the binding surface on UNC-78. Instead, the mutation appears to inhibit activation of filament disassembly. These results suggest that the outer regions of blade 3 and 4 contribute to a functional surface of UNC-78 that is required for binding to UNC-60B-F-actin and activation of filament disassembly.

E126A is a particularly interesting mutation that shows enhanced filament binding without strong filament disassembly. This mutant may indeed have stronger affinity with UNC-60B-F-actin than wild type. Alternatively, the mutant may bind to UNC-60B-F-actin in a similar manner to wild type, but loss of the disassembly activity of the mutant may make the filament binding more apparent. If this is the case, binding of UNC-78 to the UNC-60B-F-actin complex might be an intermediate step during the disassembly process. Although we were not able to detect saturable binding of wild type UNC-78 to UNC-60B-F-actin, Xenopus AIP1 has been shown to co-sediment with ADF/cofilin-bound F-actin at a 1:2 stoichiometry (8), which is consistent with the binding of the E126A mutant to UNC-60B-F-actin. Because of stable association of this mutant with UNC-60B-F-actin, the E126A mutant might be suitable for structural analysis of the actin filaments decorated with UNC-60B and UNC-78 (E126A) by cryoelectron microscopy, which will allow us to model how UNC-78 might interact with UNC-60B-F-actin. Interestingly, in Arabidopsis AIP1s, Glu126 is not conserved, but glycine is used at the equivalent position (Supplemental Fig. 1). In vitro, Arabidopsis AIP1 has activity to disassemble rabbit actin filaments decorated by lily pollen ADF (27), whereas AIP1 strongly co-localizes with ADF to filamentous actin structures in pollen grains (27). Therefore, Arabidopsis AIP1 may have different biochemical properties from other AIP1s.

In contrast, the simple reduction in the activity of the D168A, F182A, and F192A mutants suggests that these residues are required for activation of disassembly, whereas enhanced activity of K181A suggests a negative role of this residue in this function. The competing contributions of these residues are likely to generate a molecule with the biologically optimal activity, which need not be the maximal achievable biochemical activity. D168A, F182A, and F192A showed nearly normal activity to bind to UNC-60B-bound F-actin, indicating that the reduction in the disassembly activity is not simply due to reduced affinity with the filaments. It is noteworthy that the functionally important residues identified in UNC-78/AIP1 cluster closely on the surface as compared with relatively wide distribution of actin residues in subdomains 3 and 4 that are required for the two-hybrid interaction with yeast AIP1 (6). The AIP1-interacting residues in subdomain 3 are also required for interaction with cofilin (9), suggesting that AIP1 directly interacts with subdomain 4 of actin and indirectly with subdomain 3, which might be mediated by endogenously expressed yeast cofilin. Thus, if UNC-78/AIP1 interacts with both actin and ADF/cofilin, another functional surface must exist on UNC-78/AIP1. Such a second active site may be present in propeller 2 of UNC-78/AIP1, which was not extensively mutagenized in this study. Mutagenesis studies in yeast AIP1 indeed suggest the existence of the second active site in propeller 2.

The next important question is whether the mutant UNC-78 proteins with altered activity can function in vivo. It is particularly interesting to determine biological significance of the filament disassembly and capping activities. The unc-78-null worms exhibit severe disorganization of the actin filaments in body wall muscle, and four point mutations were found to cause less severe phenotypes than the null mutant (21). These point mutations are mapped to distant positions from blades 3 and 4 (Fig. 7). However, bacterially expressed recombinant UNC-78 proteins with these mutations were insoluble, and we were not able to characterize their activities. It is likely that these mutations affect the conformation of the protein and alter activity. Our preliminary experiments indicate that expression of wild type UNC-78 fused with green fluorescent protein in the unc-78-null worms can rescue the mutant phenotype and restore organized actin filaments. With this system, we will be able to test in vivo activities of the mutant UNC-78 proteins.

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Identification of Functional Residues on Caenorhabditis elegans Actin-interacting Protein 1 (UNC-78) for Disassembly of Actin Depolymerizing Factor/Cofilin-bound Actin Filaments

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