A Bacterial Toxin Disrupts Actin Filament Packing
A Steric Antagonism of Actin Polymerization by a Salmonella Virulence Protein

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Summary
Salmonella spp. require the ADP-ribosyltransferase activity of the SpvB protein for intracellular growth and systemic virulence. SpvB covalently modifies actin, causing cytoskeletal disruption and apoptosis. We report here the crystal structure of the catalytic domain of SpvB, and we show by mass spectrometric analysis that SpvB modifies actin at Arg177, inhibiting its ATPase activity. We also describe two crystal structures of SpvB-modified, polymerization-deficient actin. These structures reveal that ADP-ribosylation does not lead to dramatic conformational changes in actin, suggesting a model in which this large family of toxins inhibits actin polymerization primarily through steric disruption of intrafilament contacts.

Introduction
Salmonella spp. cause billions of human and agricultural infections each year, leading to over three million human deaths due to typhoid fever alone (Pang et al., 1995). The spread of multidrug-resistant strains of Salmonella and biosecurity concerns have recently focused more attention on this widespread pathogen (Angulo et al., 2000; Cohen and Tauxe, 1986; Holmberg et al., 1984; Tacket et al., 1985; Torok et al., 1997). Salmonella species utilize two virulence-associated type III protein secretion systems (contained in the pathogenicity islands SPI-1 and SPI-2) to inject virulence factors into host cells (Galan, 2001; Hansen-Wester and Hensel, 2001). SPI-1 functions in the early stages of infection, inducing bacterial uptake into nonphagocytic cells of the intestine (Galan and Zhou, 2000). SPI-2 is involved subsequently in systemic infection, allowing the bacteria to form a replicative niche (the Salmonella containing vacuole, or SCV) inside host cells and to colonize tissues beyond the intestine (Hensel, 2000; Waterman and Holden, 2003).

Intracellular replication of several strains of Salmonella in human and animal cell lines, as well as systemic infection in animal models, requires the spv virulence locus (plasmid or chromosomally located) and, in particular, the SpvB gene (Caldwell and Gulig, 1991; Libby et al., 2002; Matsui et al., 2001). Although controversy exists regarding whether SpvB is delivered via an SPI-2-dependent translocation process (Browne et al., 2002; Gotoh et al., 2003; Guiney and Lesnick, 2005), this virulence factor exerts its function subsequent to the actin reorganizing effects of both SPI-1 invasion-associated effectors and those of the SPI-2 SCV-related effectors (Guiney and Lesnick, 2005). Expressed alone in mammalian cells or delivered by pathogenic bacteria, SpvB disrupts the host cell cytoskeleton, culminating in a cell death with many features of apoptosis (Browne et al., 2002). It has been hypothesized that this apoptotic death may facilitate pathogen spread to fresh cells through autophagy (Guiney and Lesnick, 2005). Its important role in virulence makes SpvB an attractive target for antibacterial development.

Several studies have shown that SpvB possesses active site similarities to the large toxin family of mono-ADP-ribosyltransferases (ATRs) that covalently transfer ADP-ribose (ADPR) from nicotinamide adenine dinucleotide (NAD) to host proteins in order to modify their activity (Lesnick et al., 2001). SpvB was recently shown to ADP-ribosylate actin, and thereby to inhibit the polymerization of this central cytoskeletal protein (Tezcan-Merdol, 2001), placing it in a subclass of ATRs targeting actin directly and indirectly, which includes Clostridium botulinum C2 (iota toxin), Bacillus cereus vegetative insecticidal protein (VIP2), C. perfringens iota toxin, C. spiriforme toxin, and C. difficile toxin (Barbieri et al., 2002; Barth et al., 2004; Han and Tainer, 2002; Pallen et al., 2001).

Mono-ADP-ribosylation occurs in eukaryotic cells in conjunction with ADP-ribosylhydrolases to modulate the activity of proteins, and it represents a relatively poorly characterized biochemical regulatory process affecting signal transduction and metabolism (Corda and Di Girolamo, 2003). A large number of bacteria use ATRs as cellular toxins to induce irreversible changes through modifications that cannot be removed by host enzymes (Pallen et al., 2001). We have examined SpvB biochemically and through X-ray crystallography in order to understand its biological activity. We also present studies of its host target protein, actin, identifying the site of ADP-ribosylation and determining crystal structures of SpvB-treated actin. These studies have led us to a model for the mechanism of filament disruption caused by SpvB within the host cell.

Results and Discussion
A minimal ATR domain of SpvB was defined by limited proteolysis (spanning residues 390–591), and its activity was verified in actin polymerization assays (Figure 1 and Experimental Procedures). Nucleotide-free and NADH (nonhydrolyzable form of NAD) structures were determined to 1.5 Å and 1.9 Å resolution, respectively (Figures 1 and 2A–2C; Table 1). These structures reveal that the SpvB ATR domain adopts the canonical fold of this broad toxin family, consisting of an a/b fold with two nearly perpendicular β sheets juxtaposed at a helical
domain, and aligns best to VIP2 and C. botulinum C3 with root-mean-square deviations (rmsds) in the Ca positions of 2.4 Å and 2.7 Å over 140 residues (with Z scores of 13.4 and 12.4, respectively; Holm and Sander, 1993) (Figures 2A and 3A). The key active site residues, SpvB Arg471 and Glu538, are located in very similar positions to homologous residues in a subclass of ATRs, including VIP2 and C3, that make critical contacts with NAD by using an arginine instead of a histidine (the histidine contact is found in the diphtheria ATR toxin family) (Han et al., 1999, 2001). The SpvB ATR domain differs from the other actin targeting enzymes (and all ATRs known) by a 30 amino acid insertion in the helical subdomain (SpvB residues 415–445) (Figure 2) as well as by its molecular surface (Figure 3B).

The cocrystal structure of the SpvB ADR domain with NADH reveals that SpvB binds NAD in a manner similar to VIP2 and C3 (Figure 3). Overall, SpvB, like VIP2, makes a smaller number of contacts to the cofactor than does C3. Like C3 and VIP2, SpvB uses an arginine residue (Arg414) to interact with phosphate oxygen atoms of NADH (Figure 3C). The active site catalytic residues of SpvB, Arg471 and Glu538, also make contacts to the ribose and phosphate oxygen atoms, as do homologous residues in the other ATR toxins. These key similarities indicate that SpvB will function enzymatically in a manner very similar to previously described members of the ATR family.

Several actin targeting ATRs have been characterized, and in two cases the site of modification has been mapped to Arg177 (Vandekerckhove et al., 1987, 1988). Mechanistic models for other enzymes, such as VIP2, have been published with this residue as the assumed target (Han et al., 2001). Using mass spectrometric analyses, we show that actin, both muscle and non-muscle actin, is mono-ADP-ribosylated by SpvB, and that, indeed, this modification is at Arg177 (Figure 4; Tables S1–S4 in the Supplemental Data available with this article online). Intact molecular weight analysis of the unmodified and SpvB-modified actin produced molecular weights differing by ~ 544 Da, consistent with the addition of an ADPR group (541 Da). Trypsin digestion and subsequent LC/FTMS analysis (combined liquid chromatography and Fourier transform mass spectroscopy) of the ADPR-actin was used to identify the site of modification. Since peptides with an ADPR-arginine residue at the C-terminal end are not susceptible to tryptic cleavage (Zhou et al., 1996), an SpvB-modified peptide should contain an (unmodified) arginine cut at the C terminus and an ADPR-arginine within the interior sequence of the peptide. Automated analysis of the accurate mass (5 ppm window) data acquired in this experiment showed the presence of peptides that cover 93% of the sequence (Figure 4; Tables S1–S4). Further automated analysis of the LC/FTMS data was performed by searching the data set for all possible tryptic fragments, with up to two missed cleavages, with an additional mass of 541.0611 Da, which corresponds to ADP-ribosylation. This procedure identified a tryptic peptide spanning residues 148–183 containing ADPR, MW = 4363.0259 Da (theory) and MW = 4363.0280 Da (observed). Subsequent LC/FT MS/MS analysis of this peptide confirmed the presence of ADP-ribosylation.
The only arginine residue within this sequence is Arg177 (discounting Arg183, which is the C-terminal residue and the site of tryptic digest). These results are consistent with recently published data with the actin homolog, Act88F (Drosophila indirect flight muscle protein), in which the mutant of Act88F, Arg177Gln, showed...
only marginal ribosylation in relation to wild-type when treated with SpvB (Hochmann et al., 2006).

Despite the knowledge that Arg177 is targeted by this broad family of toxins, now including SpvB, it is not well understood how this modification prevents polymerization. Previously, it has been shown that ADP-ribosylated actin at Arg177 acts as a capping protein, capping the barbed ends and impeding the addition of new actin monomers, leading ultimately to inhibition of polymerization at substoichiometric ratios (Wegner and Aktories, 1988). ADPR-actin has no effect on the rate of polymerization of gelsolin-capped actin filaments that polymerize at the pointed ends, but it inhibits the polymerization at the barbed ends, suggesting that it cannot bind to and cap the pointed ends of the filaments (Wegner and Aktories, 1988). Microinjection of Arg177 ADPR-actin caused actin cytoskeletal disruption even in the absence of an ATR, indicating that it is the modified actin alone that confers the cytotoxic effects (Kiefer et al., 1996). How these effects can be explained by the modification at Arg177 has remained unresolved.

Two explanations for the effect of Arg177 ADP-ribosylation, not mutually exclusive, appear likely: (1) that this modification induces conformational changes in actin not compatible with filament formation, and (2) that the presence of ADPR at Arg177 causes a steric clash preventing protein-protein interactions necessary for polymerization. In order to differentiate between these possibilities, we determined the structure of actin that had been ADP-ribosylated with SpvB (Table 2 and Experimental Procedures). To date, all crystal structures of actin have been in the G form, either bound to proteins that prevent polymerization (such as DNase I) or inhibited pharmacologically from polymerizing, such as by the drugs TMR (tetramethylrhodamine-5-maleimide) or latrunculin A (Bubb et al., 2002; Graceffa and Dominguez, 2003; Morton et al., 2000; Otterbein et al., 2001). The filamentous forms of actin have resisted all attempts at high-resolution structural determination. Crystallization in the case of SpvB was feasible because SpvB-treated actin is resistant to polymerization, even at the high protein concentrations required for crystallization. Two different crystal forms of actin, possessing very different crystal packing arrangements, were obtained. In one, actin crystallizes as a monomer in a hexagonal lattice, whereas in the other, similar to what was seen in actin crystals with latrunculin A and polylysine (Bubb et al., 2002), two actin molecules form an antiparallel dimer in the asymmetric unit in an orthorhombic lattice.

The three independent structures of actin in these two crystal forms are very similar, and they align with rmsds in Cx positions of less than 0.4 Å for all actin residues (Figure 5A). The hexagonal structure aligns well to the two orthorhombic molecules, but there is a slight

Figure 3. Cocrystal Structure of SpvB with NADH

(A) Alignments of SpvB with other ADP-ribosyltransferases based on minimizing the Cx positions of the core fold (SpvB yellow subdomain). (B) Molecular surfaces of SpvB and the Bacillus cerus vegetative insecticidal protein (VIP2) colored by relative electrostatic potential (red, negative or acidic; blue, basic or positive). The insertion in SpvB is indicated. The cofactor is shown as a chemical model binding in the active site pockets of the enzymes.

(C) Schematic of the contacts to NADH from SpvB. Solid, green bonds indicate the NADH molecule, and black bonds indicate residues from the protein. Hydrogen bonds are shown as green, dashed lines between atoms (and the distance of the bond in angstroms is noted). van der Waals contacts are shown in brown. Carbon, nitrogen, oxygen, and phosphorous atoms are shown in black, blue, red, and magenta, respectively. Residues in SpvB are labeled and numbered.
Structure of Salmonella SpvB and Modified Actin

Figure 4. Mass Spectrometric Analysis and Mapping of the ADPR Site

(A) Top panel: extracted mass chromatogram of the (M+3H)+ ion for ADPR-modified peptides 148–183 in a control tryptic digest. Second panel: extracted mass chromatogram of the (M+3H)+ ion for ADPR-modified peptides 148–183 in a digest of ADPR-actin. Third panel: mass chromatogram for the (M+4H)+ ion for ADPR-modified peptides 148–183 in a tryptic digest of actin from crystals. Fourth panel: mass chromatogram for the (M+3H)+ ion for unmodified peptides 143–177 in actin from crystals.

(B) Partial mass spectra acquired by LC/FTMS for the (M+4H)+ ions for ADPR-modified actin. Top panel: theory for C_{184}H_{290}N_{52}O_{65}P_{2}S_{1}. Middle panel: acquired data for ADPR-actin. Bottom panel: acquired data for ADPR-modified actin from crystals. Note that all accurate mass data matched theoretically to < 2 ppm mass deviation.

(C) Spectrum acquired via LC/FTMSMS of the ADPR-modified actin tryptic digest showing daughter ions of 1092, verifying assignment as (M+4H)+ of ADP-modified peptides 148–183.


(E) Partial mass spectrum from LC/FTMS of the tryptic digest of nonmuscle actin of (M+5H)+. Top panel: theoretical (C_{186}H_{291}N_{51}O_{65}P_{2}S_{1}). Bottom panel: acquired data.
displacement in residues 55–68 (immediately after the DNase I binding loop) and in residues 226–237. Between the two copies of actin in the orthorhombic asymmetric unit, there is a relative opening-closing hinge motion in the nucleotide binding cleft of actin. In all three cases, the DNase I binding loop (residues 39–51) is disordered, and the nucleotide in the active site in all three structures is the natural ligand, ATP (Figure 5B). The covalent modification, ADPR, is not visible in any of the electron density maps, suggesting that it is highly flexible and disordered. Arg177 is not disordered, but its temperature factors are somewhat higher than the average for the protein (hexagonal crystal form: overall temperature factor = 18 Å²; orthorhombic, dimeric, crystal form: overall B = ~30 Å²), with the guanidinium group the highest of all (hexagonal crystals temperature factor = ~40 Å²; orthorhombic = ~60 Å²). The crystal packing in both forms is such that Arg177, and in fact the entire surface of actin on which it is located, makes no crystal packing contacts. The residue is solvated, providing ample space for a flexible ADPR moiety.

The lack of interpretable density for the ADPR group prompted us to examine the status of this modification in the crystals. SpvB ADP-ribosylated actin was crystallized, and the protein crystals were harvested, dissolved, and analyzed by mass spectrometry (Figure 4A, third and fourth panels). The results of this analysis indicate the presence of components, of approximately equal abundance, including residue Arg177 with and without modification. This partial modification is likely due to a hydrolysis of the modifying group during the 2 week period that was needed for crystal growth. The peaks in the mass spectrometry are not quantitative because the two Arg177-containing peptides are different, and it is difficult to ascertain the relative amounts of modified and unmodified actin. Material analyzed immediately after removal from cryogenic storage, however, provides a single peak with the mass corresponding to ADP-ribosylated actin. Since the crystals appear in a day and require a few days for growth, it was technically feasible to trap actin in a highly modified state in crystals. However, hydrolysis does occur to a significant extent in the crystals, and it is favored by the high salt concentration of the crystallization solution. Thus, partial

<table>
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<th>Table 2. ADPR-Actin Data Collection and Refinement Statistics</th>
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<td><strong>Hexagonal</strong></td>
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R_sym = \( \sum h_i |I_{h,i} - \bar{I}_h| / \sum h_i I_{h,i} \) for the intensity (I) of i observations of reflection h. Values in parentheses are for the high-resolution shell. R = \( \sum |F_p| - |F_o| / \sum |F_p| \) is the experimental structure factor, and F_p is the model structure factor and 5% data omitted for R_free.

![Figure 5. Two Crystal Structures of ADP-Ribosylated Actin](image)
occupancy, possibly in conjunction with disorder, likely explains the inability to visualize this ADPR moiety.

Overall, the structures of actin obtained reveal that there are no large-scale conformation alterations in actin upon ribosylation by SpvB (Figure 5C), which argues that such allosteric changes are not the primary cause of polymerization deficiency induced by this modification. The mixture of modified and unmodified actin in the crystals discussed above likely does not invalidate this conclusion. To begin, even if some fraction (less than 50%, as judged by the relative peak heights) of the actin in the crystalline form remains ADP-ribosylated, the fact remains that all actin molecules in the crystals have the same structure. It is unlikely that crystal packing could accommodate two significantly different structures for the two forms of actin. That we have two completely different crystal forms of actin, with a total of three independent molecules of actin between them, supports this conclusion. Were such discordant crystal packing even to be the case, the low occupancy of the ADP-ribosylated form would likely lead to extremely weak electron density for the differing polypeptide regions. The only place this could have reasonably occurred, therefore, is the DNase I binding loop, which is disordered. However, disorder in this region is the norm for the ATP bound form of actin (Kudryashov and Reisler, 2003).

Therefore, the model of a steric clash-induced hindrance of intraprotomer contacts in the filament likely provides the best explanation for the activity of this virulence factor, and by extension, other bacterial toxins that target Arg177 for ADP-ribosylation. In the filament model of Holmes et al. (1990), Arg177 is proximal to Glu195, Ser199, Phe200, and Val201 of a protomer in the opposing strand of the filament, and it likely interacts with these residues, leading to cross-strand stabilization within the actin helix (Figure 6).

ADPR at Arg177 would lead to a dramatic steric clash at the contact point between actin subunits and, therefore, to unfavorable protomer-protomer contacts (Figure 6). This is consistent with data showing that Arg177 is expected to be completely buried upon the formation of the Holmes model of actin, based on calculated solvent-accessible surface area (ASA) changes upon the formation of F-actin and footprinting with hydroxyl radicals generated by synchrotron X-ray radiolysis (Guan, 2005). This is further supported by the finding that a 2.6 kDa peptide of actin extending from Arg177 to Tyr198 binds tightly to actin (Hori and Morita, 1992) and by a large amount of mutagenesis showing that various mutations in Arg177 lead to developmental deformities in fish, cytoskeletal abnormalities in yeast, and in vitro and in vivo impairment of actin nucleation and polymerization (Drubin et al., 1993; Ng and Abelson, 1980; Schuler et al., 2000; Wong et al., 2001).

Because of the extended time period required to grow diffraction-quality crystals, the presence of ATP in the active site of both crystal forms (indicating a lack of ATP hydrolysis) suggested that ADP-ribosylation of actin by SpvB may inhibit the intrinsic ATPase activity of actin, as has been observed in studies of actin modified by other toxins. This indeed was found to be the case in biochemical assays (Figure 7). An assay that measures the release of inorganic phosphate from actin during

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**Figure 6. Steric Clash Due to ADPR in the Actin Helix**

The Holmes model for the actin filament is shown with the actin protomers differentially colored. Arg177 is shown in blue in a space-filling representation. Sequential magnifications of the helix are shown, and the final magnification illustrates the putative Arg177 interaction residues Glu195, Ser199, Phe200, and Val201. Superimposed on this last image is the space-filling representation of ADP-ribose (red) to illustrate its relative size and emphasize the steric clash that would occur were it conjugated to Arg177.

**Figure 7. SpvB-Mediated ADP-Ribosylation Inhibits Phosphate Release in Actin**

The inorganic phosphate released continuously in solution by the intrinsic ATPase activity of actin was measured by an increase in absorbance (Experimental Procedures), shown in relative absorbance units, for the ribosylated actin and for the control reaction containing actin.
F-actin polymerization reveals that the rate of γ-phosphate release is decreased from ADP-ribosylated actin. Arg177, conserved in all actin homologs, is considered to be involved in the γ-phosphate release from ATP, although the precise mechanism is not well understood. Molecular dynamics modeling suggested a “backdoor” mechanism in which the formation of a salt bridge between MeHis73 (methylated histidine) and Asp184 creates a state of phosphate release and in which Arg177 significantly retards the release of the phosphate by interacting with it (Wriggers and Schulten, 1997, 1999).

Salmonella spp. utilize an arsenal of bacterial virulence factors to modulate the host cell cytoskeleton (Galan, 2001). Many of these, such as those of the RhoGTPase family, indirectly do so by manipulating signal transduction cascades (Lilic and Stebbins, 2004; Schlumberger and Hardt, 2005). Others directly modulate cytoskeletal structure by binding to and interacting with cytoskeletal proteins such as actin and tubulin (Gruenheid and Finlay, 2003; Stebbins, 2004). A large number of these factors function in the initial stages of infection through the SPI-1 pathogenicity island on the bacterial chromosome by inducing actin polymerization, leading to membrane ruffling and bacterial internalization (Galan, 1999). The SPI-2 pathogenicity island functions during later stages of infection, and it also interacts with the host cytoskeleton to maintain the specialized Salmonella-containing vacuole. SpvB inhibits actin polymerization through mono-ADP-ribosylation of actin, leading to apoptosis and potentially thereby contributing to bacterial spread to other professional phagocytes upon autophagy (Guiney and Lesnick, 2005). Consistent with this, SpvB is a critical element in systemic virulence during infection with Salmonella.

We have shown that SpvB is a divergent ATR in that it possesses, to our knowledge, novel elements in the fold. However, its close similarities in the active site and cofactor binding indicate that, as an enzyme, it likely functions in a similar fashion to other actin-modifying ATRs in targeting actin. Using mass spectrometry, we were able to clearly establish that actin is ADP-ribosylated at Arg177, the same residue of actin that is found to be modified by several other ATR enzymes targeting this cytoskeletal protein.

Few structures of actin are available due to its propensity to polymerize. While structures of monomeric actin in complex with proteins that sequester it from filament formation or that are prevented from polymerizing through pharmacological alteration exist, this report presents, to our knowledge, the first structures of actin that have been modified by a bacterial toxin. We have found that the ADP-ribosylation of actin by SpvB at Arg177 inhibits ATPase activity and likely prevents polymerization through a steric mechanism that involves impeding critical protomer-protomer contacts required for filament formation and stability. SpvB-treated and -crystallized actin does not show any major conformational alterations relative to other monomeric actin structures, which therefore suggests that large-scale conformational changes due to this modification likely cannot explain the defect in polymerization. However, an analysis of the protomer packing of actin in the filament indicates that the presence of ADPR at Arg177 would lead to severe steric clash and likely to a disruption of the contacts modeled to hold the actin filament together.

Experimental Procedures

Protein Purification and Domain Delineation of SpvB
SpvB (residues 370–591) was cloned by PCR from the Salmonella typhimurium virulence plasmid and expressed in Escherichia coli BL21(DE3) cells as N-terminal His, fusion protein by using the pET28 expression vector. The protein was purified by nickel ion affinity chromatography and was then subjected to limited proteolysis with subtilisin (Figure 1A). Fragments identified through N-terminal sequencing and mass spectrometry were subcloned into the same vector and purified as described above. A stable fragment spanning residues 390–591 was active in actin ribosylation assays (Figure 1B) and produced crystals. Selenomethionine-substituted protein was prepared by following standard protocols (Doublié, 1997). Both, wild-type (SpvB) and the selenomethionine (Se-SpvB) proteins were purified to homogeneity by nickel ion affinity chromatography, followed by the removal of the tag with thrombin, subtractive nickel ion-Sepharose, and gel filtration chromatography on Superdex 75 (GE HealthCare). For crystalization, the purified SpvB and Se-SpvB proteins were concentrated to 80 and 60 mg/ml, respectively, by ultrafiltration in 100 mM NaCl, 10 mM Tris–HCl (pH 8), and 2 mM DTT.

Actin Ribosylation
Actin (Cytoskeleton, Inc.) at 1–2 mg/ml in buffer containing 5 mM Tris (pH 8.0), 0.2 mM ATP, 0.2 mM CaCl2, 2 mM DTT was mixed with SpvB protein (100 μg), NAD (200 μM final concentration). After overnight incubation at 4°C, 100 μg SpvB protein and polymerization buffer to a final concentration of 50 mM KCl, 2 mM MgCl2. 0.2 mM ATP were added to this mixture, and the mixture was incubated at room temperature for 2 hr. After incubation, the actin sample was centrifuged at 100,000 × g for 1 hr at 4°C to separate G-actin, presumably ribosylated, from F-actin. The supernatant was purified further on a HiLoad Superdex 75 gel filtration column in a buffer containing 5 mM Tris (pH 8), 0.2 mM ATP, 0.2 mM CaCl2, 2 mM DTT, and 100 mM NaCl. For crystallization, the ribosylated actin was concentrated at 15–30 mg/ml by ultrafiltration in buffer containing 5 mM Tris (pH 8), 0.2 mM ATP, 0.2 mM CaCl2, and 2 mM DTT and was flash frozen in liquid nitrogen for storage.

Protein Crystallization
SpvB and Se-SpvB crystals were obtained via hanging-drop vapor diffusion against a reservoir solution consisting of 15% PEG 4000, 0.2 M Li2SO4, 100 mM Tris–HCl (pH 8.5), 4% sucrose, at 4°C for 1 hr. After overnight incubation at 4°C, 100 μg SpvB protein and polymerization buffer to a final concentration of 50 mM KCl, 2 mM MgCl2. 0.2 mM ATP were added to this mixture, and the mixture was incubated at room temperature for 2 hr. After incubation, the actin sample was centrifuged at 100,000 × g for 1 hr at 4°C to separate G-actin, presumably ribosylated, from F-actin. The supernatant was purified further on a HiLoad Superdex 75 gel filtration column in a buffer containing 5 mM Tris (pH 8), 0.2 mM ATP, 0.2 mM CaCl2, 2 mM DTT, and 100 mM NaCl. For crystallization, the ribosylated actin was concentrated at 15–30 mg/ml by ultrafiltration in buffer containing 5 mM Tris (pH 8), 0.2 mM ATP, 0.2 mM CaCl2, and 2 mM DTT and was flash frozen in liquid nitrogen for storage.

ADP-ribosyl-actin (ADPR-actin) crystals were obtained by soaking SpvB crystals overnight in the reservoir solution containing 1.5 mM Na2SO4, 0.2 M Li2SO4, 100 mM Tris–HCl (pH 8.5), 4% sucrose, at 4°C, by using a protein concentration of 20 mg/ml. Crystals of SpvB and Se-SpvB grow spontaneously; however, their size and morphology were improved dramatically by using microseeding. Crystal cryoprotection was achieved by adding PEG 1000 in the crystallization drop to a final concentration of 17.5% (v/v). Crystals diffracting to 1.5 Å (SpvB) and 2.0 Å resolution (Se-SpvB) grew in the hexagonal space group P321 (a = b = 55.5 Å, c = 143.5 Å; α = β = γ = 90 °) with one molecule in the asymmetric unit. All crystals appeared in 2–3 days and grew to full size within 2–3 weeks. Crystals of SpvB-NADH were obtained by soaking SpvB crystals overnight in the reservoir solution containing 1.5 mM NADH. SpvB-NADH crystals were cryoprotected by adding PEG 1000 in the crystallization drop to a final concentration of 17.5% (v/v) as well as 5 mM NADH.

ADP-ribosyl-actin (ADPR-actin) crystals were obtained by hanging-drop vapor diffusion against a reservoir solution consisting of 1.25 M (NH4)2SO4, 0.2 M Li2SO4, 50 mM Tris–HCl (pH 8.5) (hexagonal form), or against a reservoir solution consisting of 1 M (NH4)2HPO4, 0.2 M NaCl, 100 mM citrate buffer (pH 5.5) (orthorhombic form), at 20°C, by using a protein concentration of 10 mg/ml. Crystals of ADPR-actin grew spontaneously in both conditions; however, their size and morphology was improved by using microseeding. Crystal cryoprotection was achieved by adding ethylene glycol in the crystallization drop to a final concentration of 25% (v/v) for the hexagonal form and glycerol to a final concentration of 25% (v/v) for the orthorhombic form. Crystals diffracting to 1.7 Å resolution grew in the hexagonal space group P3211 (a = b = 96.36 Å, c = 97.41 Å; α = β = γ = 90, α = β = γ = 90).
γ = 120) with one molecule per asymmetric unit in the sulfate solution. Crystals diffracting to 2.0 Å resolution grew in the orthorhombic space group P212121 (a = 100.38 Å, b = 102.34 Å, c = 123.8 Å; β = γ = 90) with two molecules per asymmetric unit in the phosphate solution.

Data Collection, Structure Determination, and Refinement

Diffraction data for all five structural determinations were measured at beamlines X9A or X29 of the National Synchrotron Light Source at Brookhaven National Laboratory. A single-wavelength anomalous dispersion experiment (SAD) was conducted at an X-ray wavelength near the selenium absorption peak with a single SpvB crystal at the beamline X9A. Data were processed with the HKL software package (Otwinowski and Minor, 1997). All four Se sites were found by using SOLVE (Terwilliger, 1999; Terwilliger and Berendzen, 1999). The experimental phases were improved by density modification by using RESOLVE (Terwilliger, 1999; Terwilliger and Berendzen, 1999), yielding interpretable electron density maps of high quality to 2.0 Å. Roughly 85% of the protein main chain was built automatically by this program. This partial structure was positioned in the native crystallized data set by molecular replacement with AMoRe (Navaza, 1994; Otwinowski and Minor, 1997). All refinements were performed with O and REFMAC5, producing final models with R/Rfree of 17.1/21.0% to 2.0 Å for the orthorhombic crystal form.

Actin Polymerization Assay

Pyrene G-actin (Cytoskeleton, Inc.) in buffer containing 5 mM Tris (pH 8), 0.2 mM ATP, 0.2 mM CaCl2 was preclarified by centrifugation at 100,000 × g before use. For the actin polymerization assay, pyrene G-actin (10 μM) in buffer containing 5 mM Tris-HCl (pH 8.0), 0.2 mM CaCl2, 0.2 mM ATP was mixed with NAD (200 μM) and with SpvB (2 μM) in buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM DTT. The polymerization of G-actin was initiated by the addition of polymerization buffer at a final concentration of 50 mM KCl, 2 mM MgCl2, 0.2 mM ATP. Fluorescence intensity was subsequently measured on a fluorescence spectrophotometer (SpectraMAX Gemini) with the excitation wavelength set to 365 nm and the emission wavelength set to 407 nm. To determine the inhibitory activity of SpvB by the mono-ADPR transferase-specific inhibitor, novobiocin, at a final concentration of 200 μM, was added to the polymerization mixture containing SpvB and NAD. As a control, polymerization reactions without SpvB were run in parallel.

Phosphate Release Assay

The inorganic phosphate released from F-actin during polymerization was measured in a coupled assay by using the EnzChek Phosphate Assay (Molecular Probes) for stopped-flow kinetic experiments. The method consists of an enzymatic conversion of the 2-amino-6-mercapto-7-methylpurine riboside (MESG) substrate to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by purine nucleoside phosphorylase (PNP) in the presence of Pn. The conversion is accompanied by a change in absorption at 360 nm. The assay was used to follow the kinetics of the Pn released continuously by the ATPase activity of actin versus the ADP-ribosylated actin. Briefly, 400 μg actin or SpvB-modified actin was incubated with 1 unit of PNP enzyme, 200 μM MESG in 50 mM Tris (pH 7.5), 1 mM MgCl2, 0.01 sodium azide for 30 min at 22 °C, and the absorbance at 360 nm was read as a function of time.

Mass Spectrometric Analysis of ADP-Ribosylated Actin

For intact molecular weight analysis, 5 μl of 2 mg/ml actin samples were injected onto a 150 × 1 mm Jupiter C4 column (Phenomenex) and eluted with a linear gradient with direct flow into a Micromass Quattro Ultima mass spectrometer (Waters) operated under positive ESI conditions. The acquired data were deconvoluted with MaxEnt1 to yield the mass of the protein. For the identification of the ADP-ribosylation site, the ADP-ribosylated actin samples were digested overnight with a 50:1 actin:trypsin ratio at 37 °C. The digested samples were analyzed by HPLC FTMS. The peptides (10 μl) were injected onto a 150 × 1 mm Hypersil C18 column (Thermo) and eluted with a linear gradient with direct flow into a Bruker APEX Q T7 mass spectrometer (Bruker Daltonics) operated under positive ESI mode. External calibration with porcine rennin substrate provided better than 5 ppm mass measurement accuracy (0.005 amu at m/z 1000). The data were analyzed automatically with the Proqual program or manually with MaxLynx (Waters). Proqual compares the molecular weight of each tryptic fragment from the spectrum with a list of all possible tryptic fragments of actin for the correct mass (within 5 ppm). Additionally, the data were searched for ADP-ribosylation by adding the mass of 541 for this specific modification to each of possible fragments.

Supplemental Data

Supplemental Data include the mass spectrometric analysis as well as additional stereo figures and are available at http://www.structure.org/cgi/content/full/14/8/1219/DC1/.

Acknowledgments

We thank H. Mueller at Rockefeller University and T. Radnakannan of Brookhaven beamline X9A for access to and assistance with crystallographic equipment, and we thank R. Bennett, C. Pepper, A. Gazes, and G. Latter of the Rockefeller University Information Technology Resource Center for computational facilities. We thank T.A. Kelly, P. Anderson, and C. Grygon from Boehringer-Ingelheim for use of the 2-amino-6-mercapto-7-methylpurine riboside (MESG) substrate provided by the 2-amino-6-mercapto-7-methylpurine riboside (MESG) substrate.

References


Accession Numbers

The coordinates for the SpvB, SpvB-NADH, hexagonal, and orthorhombic actin structures have been deposited in the PDB with ID codes 2GWM, 2GWL, 2GWJ, and 2GWK, respectively.