Photox, a Novel Actin-targeting Mono-ADP-ribosyltransferase from *Photorhabdus luminescens*

Danielle D. Visschedyk, Alexandru A. Periteanu, Zachari J. Turgeon, Robert J. Fieldhouse, John F. Dawson, and A. Rod Merrill

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*From the Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada*

*Photorhabdus luminescens* is a pathogenic bacterium that produces many toxic proteins. The mono-ADP-ribosyltransferases (mARTs) are an enzyme class produced by numerous pathogenic bacteria and participate in disease in plants and animals, including humans. Herein we report a novel mART from *P. luminescens* called Photox. This 46-kDa toxin shows high homology to other actin-targeting mARTs in hallmark catalytic regions and a similar core catalytic fold. Furthermore, Photox shows *in vivo* cytotoxic activity against yeast, with protection occurring when catalytic residues are substituted with alanine. *In vitro*, enzymatic activity (\(k_{cat} = 1680 \pm 75 \text{ min}^{-1}\)) is higher than that of the related iota toxin, and diminishes by nearly 14,000-fold following substitution of the catalytic Glu (E355A). This toxin specifically ADP-ribosylates monomeric \(\alpha\)-skeletal actin and nonmuscle \(\beta\)- and \(\gamma\)-actin at Arg\(^{177}\), inhibiting regular polymerization of actin filaments. These results indicate that Photox is indeed an ADP-ribosyltransferase, making it the newest member of the actin-targeting mART family.

Among other toxins, *Photorhabdus* bacteria produce toxin complexes, high molecular weight, multisubunit, insecticidal toxins (4), some of which show oral toxicity in the same range as *Bacillus thuringiensis* endotoxins (5), as well as the "makes caterpillars floppy" toxin, responsible for insect midgut destruction (6). Based on its pathogenesis and the high number of virulence factors that it produces, *P. luminescens* has garnered interest in the area of biopesticides due to increasing resistance against conventional pesticides (7).

Various highly pathogenic bacteria produce toxins that share the enzymatic function of covalently modifying a host protein through addition of an ADP-ribose moiety from NAD\(^+\). This covalent attachment of a bulky ADP-ribose group generally inhibits the natural function of the target protein, causing various deleterious effects within a cell. These toxins contribute to a wide variety of diseases in humans including diphtheria, pertussis, and cholera (8, 9). Historically, these mono-ADP-ribosyltransferase (mART)\(^6\) toxins have been divided into two groups: the DT group (named for diphtheria toxin) and the CT group (named for cholera toxin). Although the three known toxins of the DT group each target eukaryotic elongation factor 2, the numerous CT toxins are generally further classified depending on their targets within a host. To date, nine mART toxins have been identified that ADP-ribosylate actin and disrupt actin polymerization. Most of these are binary toxins consisting of an A component responsible for binding/translocation and a B component with mART enzymatic activity. The *Clostridium* toxins, *Clostridium perfringens* iota (10), *Clostridium botulinum* C2 toxin (11), *Clostridium sporogenes* Sa (12), and *Clostridium difficile* CDTa (13), along with *Bacillus cereus* vegetative insecticidal protein (14), function in this binary fashion. The remaining actin-targeting mARTs do not fit this architecture. *SpyA* of *Salmonella enterica* consists of a single domain, and is thought to gain cell entry via a type III secretion system (15). Likewise, *Aeromonas salmonicida* AexT uses a type III secretion system for invasion of host cells and carries a second functional domain with Rho-GAP activity (16, 17), reminiscent of the well characterized ExoS expressed by *Pseudomonas aeruginosa*. *Streptococcus pyogenes* SpyA (18) is thought to be a single-domain mART with a 30-residue signal sequence for which the mechanism of cell entry is not yet understood and most recently, VgrG1 was found to enter host cells via a type six secretion system (19).

\(^{6}\) The abbreviations used are: mART, mono-ADP-ribosyltransferase; \(\beta\)-ME, \(\beta\)-mercaptoethanol; CT, cholera toxin; DT, diphtheria toxin; \(\varepsilon\)-NAD\(^+\), etheno-NAD\(^+\); FITC, fluorescein isothiocyanate; WT, wild-type; PIPES, 1,4-piperazinediethanesulfonic acid; ADPr, ADP-ribosylated.
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Recently, the structure of a Michaelis complex with iota toxin, actin, and a non-hydrolyzable NAD$^+$ analogue was described (20). Based on this structure, Tsuge et al. (20) provided some insight into substrate recognition. In particular they were able to show that Tyr$^{62}$ on loop I and Arg$^{248}$ on loop II play an essential role at the actin-toxin interface. The authors also proposed a common reaction mechanism for the actin-targeting mART toxins whereby an oxocarbenium intermediate is formed following the cleavage of the nicotinamide moiety from NAD$^+$. Rotation then allows for the release of the conformational strain and the formation of a second cationic intermediate. Finally, the nucleophilic attack on Arg$^{177}$ of the target actin leaves the ADP-ribose group covalently bound to this target protein (20).

Because overall primary sequence identity among mART family members is most often low, identification of new members must rely on a shared core structure (SCOP code d.166.1.1.), sequence identity in several key catalytic regions, and pathogenicity of the organism as a positive indicator. In particular, a region 1 catalytic Arg (His in the DT group), preceded by an aromatic residue, aids in NAD$^+$ binding and maintaining the active site structure. In region 2 of the CT group toxins, a Ser-Thr-Ser motif on a β-strand, preceded by aromatic hydrophobic residues, forms the scaffold of the active site and stabilizes NAD$^+$ substrate binding. DT group toxins contain a Tyr-X$^{10}$-Tyr motif, playing a similar role, where X denotes any amino acid. In region 3, the catalytic Glu is found on a β-strand and is responsible for the ADP-ribose transferase activity. A second Glu or Gln is found in CT group members two residues away, and may participate in substrate recognition, but the mechanism by which this occurs is not yet known (21, 22).

Increasing our collective knowledge of the mechanism of action of this toxic enzyme family, and the description of new members will provide additional targets for antibacterial interventions and lead to successful inhibitor design against mART enzymatic activity. These efforts will undoubtedly aid in the development of antivirulence strategies and novel therapeutics for the prevention and treatment of bacterial diseases.

Herein we describe a novel putative virulence factor, Photox, produced by P. luminescens, as the newest member of the mART family. This enzyme shows primary sequence homology in key catalytic regions, and the mART overall fold in the catalytic core. It possesses ADP-ribose transferase activity and specifically targets Arg$^{177}$ of actin. Photox enzymatic activity is relatively high ($k_{cat}$ 1680 ± 75 min$^{-1}$) among actin-targeting mARTs, and it is shown to abolish actin polymerization upon covalent modification at Arg$^{177}$.

**EXPERIMENTAL PROCEDURES**

**Identification and Modeling in Silico**—The plu0822 gene was identified as a putative mART in *silico* after searching the Genomic Threading Data base (23) using SCOP code d.166.1.1 (24). The predicted fold was confirmed by metaservers 3D-JURY (25), Genesilico (26), and Pcons (27). The characteristic mART primary sequence pattern was confirmed using ScanProsite (28, 29) and multiple sequence alignments using three-dimensional Coffee (30). Photox was modeled against the SpvB-NAD$^+$ (PDB code 2GWL) using MODELLER (31), and quality assessed by MetaMQAPII (32). The Photox-actin-NAD$^+$ complex was prepared by the superposition of Photox-NAD$^+$ in place of iota toxin (PDB code 3BUZ) using Coot (33).

**Overexpression and Purification of Photox in E. coli**—The Photox gene was overexpressed in *Escherichia coli* cells and the protein was purified from inclusion bodies. In brief, the Photox gene was cloned into a pET-28b vector with an N-terminal His$_6$ tag and a tobacco etch virus protease (TEV-8) site. *E. coli* Rosetta cells were transformed with plasmid and plated onto 2× YT plates containing ampicillin to grow overnight at 37 °C. Cells were grown at 37 °C in 2-liter cultures of 2×YT containing 100 μg/ml of ampicillin to an A$_{600}$ value of ~0.6 before induction with 1 mM isopropyl 1-thio-β-d-galactopyranoside. Three hours post-induction cells were harvested by centrifugation at 5,000 × g for 10 min. Cell pellets were resuspended in 20 mM Tris–HCl, pH 7.5, 50 mM NaCl and the cells were lysed using a French press. Lysate was centrifuged for 25 min at 14,000 × g and the pellet was resuspended in 20 ml of inclusion body wash buffer (50 mM Tris–HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 0.05% deoxycholate, 0.5 mg/ml of lysozyme). After further centrifugation at 10,000 × g for 20 min the pellet was resuspended in 15 ml of denaturation buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 3 mM guanidine hydrochloride). The supernatant from a spin at 20,000 × g for 40 min was diluted to 30 ml with denaturation buffer and spiked to 5 mM imidazole. The sample was loaded to a nickel-charged chelating Sepharose column and eluted with 0–100 mM imidazole, without guanidine hydrochloride. The resulting protein was dialyzed into 10 mM Tris–HCl, pH 7.5, 100 mM NaCl and concentrated to 0.5–1.0 mg/ml using an Amicon Centriprep concentrator (Millipore, Billerica, MA).

**Purification of Actin**—Chicken skeletal α-actin was purified as described (34) and concentrations were determined by absorbance at 290 nm ($\epsilon_290 = 67,742\ M^{-1}\ cm^{-1}$). β- and γ-actin were purified using the baculovirus method described previously by Yates et al. (35). The β- and γ-actin mixture was obtained from Cytoskeleton (Denver, CO).

**Photox Substrate Assays**—Biotinylated ADP-ribosylated (ADPr) actin was detected using a one-step Western blot as described (29), with the exception of purified actin as substrate and lysate from Chinese hamster ovary cells. Fluorescein isothiocyanate (FITC)-ADP-ribose labeling of actin isoforms was obtained by incubating 19 μM FITC-NAD$^+$ with 5 μM Photox and 2 μg of purified β- or γ-actin for 1 h at 25 °C in the dark. Proteins were separated by SDS-PAGE and visualized using UV illumination and a fluorescein filter on a FluorChem 8900 (Alpha Innotech, San Leandro, CA) instrument.

**mART Kinetic Assays**—Using a stopped-flow spectrometer, model SX20-MV (Applied Photophysics, Leatherhead, UK), 80 nM Photox was mixed rapidly in a 1:1 (v/v) ratio with varying concentrations of etheno-NAD$^+$ (ε-NAD$^+$) and actin after equilibration of all samples to ambient temperature. Increasing fluorescence intensity was recorded as the reaction progressed (excitation 305 nm, emission 385 nm cut-off filter). Individual reactions were repeated with actin concentrations ranging from 0 to 15 μM and ε-NAD$^+$ concentrations ranging from 0 to 300 μM. All reactions were performed in 10 mM Tris–HCl, pH 7.5.
7.5, 0.2 mM ATP, 0.2 mM CaCl₂, β-mercaptoethanol (β-ME). A calibration curve was created by measuring fluorescence intensity changes upon completion of the reaction at various actin concentrations in the presence of excess e-NAD⁺, and by assuming the molar amounts of actin and e-NAD⁺ consumed by the reaction are equal. NAD⁺ binding and NAD⁺ glycohydrolase activities were measured as described previously (36).

**Mass Spectrometer Analysis of Modified Actin—** ADP-ribosylated α-actin was analyzed by LC/MS/MS mass spectrometry following AspN digestion to determine the site of the 541-Da ADP-ribosylation.

**Yeast Growth-Inhibition Assay—** Saccharomyces cerevisiae haploid strains W303 (MATa leu2 trp1 can1 ura3 ade2 his, DBY6945 (MATa his3 leu2 ura3 tub2 ACT1::LEU2 [pRB668(URA3)-ACT1]), and ACT-RA (MATa his3 leu2 ura3 tub2 ACT1::LEU2 [pJD301(HIS3)-ACT1 R177A]) were cultured at 30 °C on yeast-peptone-dextrose (YPD) or synthetic media.

**Phalloidin-stabilized Actin ADP-ribosylation—** Actin (10 μM, 2.5% pyrene, 200 μM NAD⁺) was polymerized for a period of 160 min in the presence or absence of a 2-fold molar excess of phallolidin. Samples were treated with 1 ng of Photox and the pyrene fluorescence intensity was measured as described previously.

**Fluorescent Yeast Labeling—** W303a yeast cells grown with 150 μg/ml of adenine to an A₅₀₀ value of 0.8 were fixed in 3.7% (v/v) formaldehyde. After centrifugation (5,000 × g, 5 min) cells were washed twice with PIPES buffer (pH 6.9, 20 mM EGTA, 20 mM MgCl₂) and resuspended in 25% (v/v) methanol in PEM buffer. After addition of 1.5 μM rhodamine phalloidin, cells were stained in the dark for ~30 min before washing and resuspension in 100 μl of PEM buffer. The 4 μl of stained cell suspension was visualized with a Nikon Eclipse 6600 epifluorescent microscope.

**RESULTS AND DISCUSSION**

Plu0822 (P. luminescens) strain TT01 was found to encode a protein of 408 residues (45.9 kDa) with high identity to other mART toxins. Although a simple BLAST search identifies this protein as a putative mART, this search strategy alone is prone to false positives and benefits from further substantiation by fold-recognition and a pattern-based search. This two-domain protein, named Photox, shares 39% primary structure identity overall with SpvB of S. enterica. Specifically, the C-terminal 200 residues of Photox share 61% identity in primary sequence with the catalytic domain of SpvB. Identity in catalytic signature regions and a predicted mART-fold indicate that plu0822 encodes a putative mART enzyme, and that Photox is likely to have enzymatic activity akin to other toxins of this family. A primary sequence alignment of Photox with several known actin-targeting mART toxins is shown in Fig. 1A. The alignment reveals strong primary sequence identity in region 1 for the catalytic Arg288 preceded by a Tyr, the region 2 Ser-Thr-Ser motif preceded by aromatic and hydrophobic residues, and region 3 primary and secondary glutamate residues, Glu355 and Glu353, respectively. Together these sequence characteristics provide strong evidence that Photox is a new mART toxin member.

**ADP-ribosyltransferase Activity Is Responsible for Cytotoxicity in Vivo—** The plu0822 gene encoding Photox was cloned into a S. cerevisiae vector under the transcriptional control of the CLP1 promoter on a low-copy number plasmid by homologous recombination, as described by Turgeon et al. (37). Basal expression of Photox in yeast cells was sufficient to incure a severe growth-defective phenotype in yeast (Fig. 1B, gray bars), demonstrating the highly toxic nature of this protein in a eukaryotic cell system. Unfortunately, complete repression of
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| Toxin, Organism | Region 1 | Region 2 | Region 3 |
|-----------------|----------|----------|----------|
| Photox, _P. luminescens_ | KKV YRLGK (24) FLSTSPD (29) GEAEML | RVVYRLGK (24) FMSTSPD (29) GEAEML | LIVYRSG (37) FISTSG (34) GEYEVL |
| SpvB, _S. typhimurium_ | LIVYRSG (37) FISTSG (34) GEYEVL | LTVYRSG (37) FIISTSG (34) GEYEVL | ITVRWC (31) YMSTSL (34) NEKIEL |
| Iota toxin, _C. perfringens_ | ITVRWC (31) YMSTSL (34) NEKIEL | LIAVRVD (43) FSSTSRL (33) DEQEL |
| CdtA, _C. difficile_ | LIAVRVD (43) FSSTSRL (33) DEQEL | |
| Vip2, _B. thuringiensis_ | |
| C2 toxin, _C. botulinum_ | |

**FIGURE 1. Identification and purification of Photox.** A, sequence alignment of several mARTs. Characteristic catalytic residues are indicated. Region 1 contains the catalytic arginine, region 2 includes the STS motif, and region 3 contains the catalytic and secondary glutamate residues. General secondary structure for these regions is indicated above the sequences (arrow denotes β-sheet). B, effects of Photox expression on yeast growth. Growth of _S. cerevisiae_ expressing WT or mutant Photox with point mutations to catalytic glutamate residues. The WT (gray), E353A (white), E355A (striped), or E353A/E355A (black) gene in pRS415 was induced with Cu²⁺ for 48 h. C, purified Photox as analyzed by SDS-PAGE and Coomassie staining. Lane 1, protein molecular mass standards (Bio-Rad) in kDa; lane 2, purified Photox.

the _CUP1_ promoter is not possible because Cu²⁺ is required for yeast cell viability and no repressor is known for this system (39). In contrast, the E353A mutation partially suppresses the lethal effects of the toxin (Fig. 1B, white bars) except at the higher Cu²⁺ concentration, confirming its accessory role in the catalytic activity of the enzyme. The effects of the E355A mutation restored yeast growth much more significantly than the E353A mutant, identifying Glu₁⁵₅ as the primary catalytic residue (Fig. 1B, striped bars). Importantly, the double mutations, E353A/E355A, almost completely abolished toxin cytotoxicity at all Cu²⁺ induction levels (Fig. 1B, black bars), indicating both residues are required for the observed mART activity in vivo and that this activity is entirely responsible for the observed growth-defective phenotype observed in yeast.

**Photox Shows mART Activity against Actin—**The _plu0822_ gene was cloned into the _E. coli_ PET-28b vector and overexpressed in _E. coli_ Rosetta cells. Photox was successfully purified from an insoluble state by simultaneous renaturation during immobilized metal affinity chromatography. The purity level and the relative mobility of the protein by SDSPAGE indicated that we had isolated the _plu0822_ protein product (Fig. 1C). Photox was conclusively identified as a His-tagged protein by Western blotting using a monoclonal antibody against the polyclonal His tag (data not shown). The yield of purified Photox was ~10 mg/liter of culture. The toxin was tested for _in vitro_ mART activity by incubating Photox with biotin-NAD⁺ and target cell lysate. ADP-ribosylated proteins carrying the biotin label (biotin-ADP-ribose) were detected on a nitrocellulose membrane following SDS-PAGE by a colorimetric reaction as described previously (40). The Photox catalyzed mART activity in the presence of biotin-NAD⁺ and purified α-actin resulted in biotin-ADP-ribose labeling of actin. A similar reaction with Chinese hamster ovary lysate resulted in a single band of the same molecular weight. The lack of other ADP-ribosylated proteins in the Chinese hamster ovary lysate indicates that actin is the primary target of this mART toxin (Fig. 2A). In addition, the substrate specificity of Photox was observed using various actin isoforms. Incubation of purified muscle α-actin or nonmuscle β- or γ-actin with Photox and FITC-NAD⁺ allowed for fluorescent visualization of ADP-riboseylated actin following SDS-PAGE. Photox proved to target each of the three actin forms as substrates in the mART reaction (Fig. 2B). Although other actin-targeting mART enzymes have been characterized using a β- and γ-actin mixture (41–43), we show here that all three purified actin isoforms individually serve as targets of this reaction. Like Photox, SpvB, Iota, Sa, and CDTa toxins have each been shown to lack specificity toward actin isoforms, which is in sharp contrast to the activity of C2 toxin, specific for β- and γ-nonmuscle forms of actin (44). Based on the structure of the iota-actin complex, Tsuge et al. (20) listed only three residues that are found at the toxin-actin interface and which are different in muscle and nonmuscle actin. One of these, Tyr²⁷⁹ of actin interacts with Tyr⁶² of iota, which was shown to be an essential residue for mART activity (20). Interestingly, a sequence alignment at this position based on PDB structure files reveals a tyrosine residue for each iota toxin, SpvB, CDTa, and Sa, but a threonine residue in C2. Therefore, this residue may play an important role in discrimination between actin isoforms. However, the N-terminal region of Photox could not be modeled accurately, and a sequence alignment at this position does not reveal any meaningful conclusions. Preliminary kinetic characterizations using purified skeletal α-actin and a mixture of β- and γ-actin showed that mART activity of Photox was only slightly higher (~3-fold, data not shown) with non-muscle actin as the substrate. As a result, in-depth kinetic characterization was conducted with α-actin as a substrate due to its availability.

**Kinetic Characterization of Photox mART Activity—**A fluorescence-based assay was used to characterize the mART activity of Photox using highly purified α-actin as protein substrate. Using a stopped-flow spectrophotometer to determine initial reaction rates, Photox (40 nm) was incubated with varying concentrations of actin and etheno-NAD⁺. In the presence of...
excess e-NAD⁺, the reaction proceeded to completion after 30 min (Fig. 2C), and a band-shift assay involving native PAGE revealed that at equilibrium nearly all of the actin is ADP-ribosylated (Fig. 2C, gel inset).

Photox mART activity exhibited Michaelis-Menten kinetic behavior with respect to the actin substrate (Fig. 2D). The Michaelis-Menten constant, $K_m$, was found to be 0.60 μM for α-actin. As shown in Table 1, Photox is a highly efficient and active mART enzyme ($k_{cat}/K_m = 10^7-10^9$ M⁻¹ min⁻¹) with a substrate turnover number, $k_{cat}$, of approximately 2200 min⁻¹. This measure of activity is comparable with that of iota toxin ($k_{cat} = 1680 ± 75$ min⁻¹) (46). Fig. 2E displays the kinetic data at various e-NAD⁺ concentrations fit to the Michaelis-Menten model. The Michaelis-Menten constant of Photox with respect to e-NAD⁺ was found to be 45 μM, which is only slightly higher than that observed for iota toxin (6.0 μM) (46).

Additionally, significant impairment in Photox mART activity was observed when signature catalytic residues were replaced with alanine. The most dramatic effect was evident when the region 1 conserved Arg was substituted with Ala (R288A showed greater than 20,000-fold reduction in $k_{cat}$; Table 2). Replacement of the primary and secondary region 3 Glu residues with Ala resulted in 13,000- and 600-fold reduction in mART activity, respectively. The conversion of the region 2 Ser-Thr-Ser motif to Ala-Thr-Ala decreased the activity by 2000-fold. These dramatic effects on mART activity indicate that these residues do indeed play an important catalytic role in the enzymatic activity of Photox, which strongly corre-
lates with other actin-targeting members of this toxin family. Previous studies have shown comparative relative activity levels for mutations in these signature catalytic residues using C2I from C. botulinum and iota toxin from C. perfringens (46, 47). Importantly, none of these mutations affected the NAD\(^{+}\) catalytic signature mutants had similar NAD\(^{+}\) binding constants (\(K_D\)) to the wild-type (WT) enzyme (Table 2).

Notably, NAD\(^{+}\)-glycohydrolase activity was not detectable for WT and mutant Photox enzymes, although most mARTs are known to possess this residual activity to hydrolyze the NAD\(^{+}\) substrate. Among actin-targeting mARTs, both C2I and iota toxin show NAD\(^{+}\)-glycohydrolase activity (43, 47), whereas SpV does not (42, 48) further highlighting the similarity of this toxin with Photox.

We used Trp fluorescence wavelength emission maximum (\(\lambda_{em}\) maximum) values to assess the folded integrity of WT and mutant Photox enzymes and the data indicated that all of the mutants were folded similar to the WT enzyme (\(\lambda_{em}\) maximum = 343–344 nm; Table 2). This was further corroborated by circular dichroism spectroscopic analysis of the proteins (supplemental Fig. S1).

**TABLE 2**
Folded stability, kinetic activity, and substrate affinity of hallmark Photox mutants

| Photox mutant | Trp \(\lambda_{em}\) maximum | Relative \(k_{cat}\) | Relative \(K_D\) |
|---------------|-----------------------------|-------------------|----------------|
| Wild-type     | 344 ± 0.6                   | 1.00              | 1.00 ± 0.03    |
| E353A         | 343 ± 0.6                   | 0.00156           | 1.22 ± 0.08    |
| E355A         | 344 ± 0.1                   | 0.000737          | 3.67 ± 0.14    |
| R288A         | 343 ± 0.1                   | ≈0*               | 4.28 ± 0.45    |
| STS/ATA       | 343 ± 0.1                   | 0.000470          | 5.41 ± 1.07    |

* Measurement could not be accurately determined as it was near background levels.

**FIGURE 3.** Photox catalyzed ADP-ribosylation on Arg\(^{77}\) of globular actin inhibits actin polymerization. A, pyrene fluorescence assay of actin polymerization. Actin (circle) or ADPr-actin (triangle) was supplemented with either G-buffer (open) or polymerization salts (closed). B, sedimentation assays of actin polymerization. Left, pellet and supernatant fractions showing >95% pelleting of actin in the presence of polymerization salts. Right, >95% ADPr-actin remains in the supernatant under similar conditions, C, effect of Photox on actin treadmilling. Pyrene fluorescence intensity of polymerized actin (×), F-actin stabilized with a 2 M excess of phalloidin to prevent treadmilling (squares), F-actin stabilized with phalloidin and treated with Photox (triangles), and F-actin treated with Photox (circles). D, growth recovery in yeast producing R177A mutant actin (gray) as compared with wild-type actin (black) when treated with Photox. Error bars show the S.D. of 8 replicates. The 100% horizontal line represents growth of yeast expressing a non-toxic aminotransferase protein, as described by Turgeon et al. (37).
completely reverse the cytotoxic effects of Photox, suggesting that ADP-ribosylation of actin at Arg177 is the only cytotoxic activity linked with Photox. The site of ADP-ribosylation on α-actin was confirmed by LC/MS/MS mass spectrometry of ADPr-actin following AspN digestion and it was revealed that the only modification site was at Arg177. Fig. 4A shows the fragmentation pattern of the ADP-ribose group. Fig. 4B displays the b- and y-ions resulting from fragmentation of the peptide containing this modification. For this peptide, b-ions are easily identifiable up to b-20, and no modifications are seen on any of these first 20 amino acids, leaving only Arg or Leu as the site of modification. Because Leu is not ionizable, the modification cannot exist here. A single site of modification on the protein is confirmed by mass spectrometry analysis of labeled and unlabeled actin show a difference of 541.28 Da (supplemental Fig. S2), equivalent to a single ADP-ribose group. Additionally, greater than 99% ADP-ribosylation efficiency was confirmed by mass spectrometry.

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To visualize the Photox-actin interactions within the cell, fluorescence microscopy of yeast cells expressing green fluorescent protein-Photox was used to observe the possible co-localization with actin (Fig. 5A). Visualization of actin using rhodamine-phalloidin resulted in images that could be overlaid with those depicting green fluorescent protein-Photox. Yellow regions of overlapping intensity indicate some co-localization of actin with Photox within the cell. Some areas where co-localization is not seen may be due to Photox association with G-actin, which would have been detected by rhodamine-phalloidin.

**Photox Homology Modeling**—Due to limited solubility, Photox proved recalcitrant to x-ray crystallography. Thus, we employed a homology modeling approach to help understand the structure-function relationships of Photox and its interactions with NAD<sup>+</sup> and actin. A model of the Photox catalytic domain (Fig. 5B) was built on the 1.9-Å x-ray structure of NAD<sup>+</sup>-bound SpvB from *Salmonella typhimurium* (PDB code 2GWL), and spans 200 residues in length. High identity (61%) between these two proteins allows for very reliable and accurate modeling. Importantly, there are no insertions or deletions in the alignment between these two proteins, and Photox includes a homologous region to the 30-residue segment previously unique to SpvB. Quality assessment on the model estimates GDT_TS at 45.62, a respectable score for homology modeling (GDT_TS stands for Global Distance Test_Total-Score and is a measure, or estimate, of the position difference between Cα atoms in theoretical and experimental models; further quality assessment can be found in supplemental Fig. S3).

The catalytic domain of Photox is an α/β protein primarily containing anti-parallel β-sheets and having separate α and β regions characteristic of the ADP-ribosylation fold (SCOP code d.166.1.1, CATH code 3.90.176.10). This model contains two sheets, two β hairpins, two β bulges, eight strands, nine helices, six helix-helix interactions, and 10 β turns. As with other members of this toxin family, NAD<sup>+</sup> binding is expected to occur through both hydrogen bonds and hydrophobic interactions. Hydrogen bonds are predicted as follows: Glu<sup>313</sup> binds to adenine, Lys<sup>291</sup> and Lys<sup>294</sup> both bind to adenine-ribose, Arg<sup>231</sup> and Asp<sup>227</sup> bind to adenine phosphate, Arg<sup>288</sup> binds to adenine and/or nicotinamide phosphate, Ser<sup>219</sup> binds to nicotinamide ribose, and Gly<sup>289</sup> binds to nicotinamide. The active site is stabilized by Ser<sup>318</sup>, which hydrogen bonds to Glu<sup>355</sup>. Thr<sup>319</sup> forms hydrogen bonds to an adjacent β-sheet to maintain active site integrity. Ser<sup>320</sup> orients the ARTT loop, including Glu<sup>353</sup>. These proposed interactions help to explain the observed decreases in activity after replacement of signature mART catalytic residues with alanine as reported above.
Gly289 was not substituted because its main chain atoms are involved in interactions within the active site, and any residue would be expected to have a similar effect. Ala replacement of those residues indicated, but not substituted in this study, are also expected to negatively affect mART activity to varying degrees based on conservation among C2- and C3-like mARTs.

The Photox homology model was assembled into a complex with actin based on the recent iota-actin 2.81-Å x-ray structure (PDB code 3BUZ). In the proposed complex, 22 Photox residues interact with 27 actin residues over an ~1,300 Å² area. Notably, Tyr223 and Phe350 of Photox are in place to potentially interact with Arg177 of actin through non-bonded van der Waals contacts.

Photox, like other mARTs, likely recognizes actin through the region B active site loop (residues 238–254), the PN loop (residues 321–329), and the ARTT loop (residues 344–354) (48). The reaction is expected to proceed through an $S_n^1$-alleviated strain mechanism (20). In this regard, Glu355 is poised to hydrogen bond to the nicotinamide ribose, whereas phosphate electrostatic interactions hold the NAD$^+$ in a conformation favoring oxocarbenium cation formation. Tyr223 is in place to possibly assist bond rotation about the nicotinamide phosphate single bond to perhaps reposition the nicotinamide ribose. Actin Asp179 may stabilize the nicotinamide ribose and Photox Glu353 is in range to stabilize actin Arg177 so it can proceed with nucleophilic attack of the oxacarbenium cation.

Using the Holmes model of filamentous actin, it has been previously shown (49) that the location of the ADP-ribosylation on actin Arg177 is an important region of intersubunit interactions. Because crystal structures of ribosylated actin monomers reveal no large structural changes due to this modification, it has been suggested that polymerization is impeded due to steric hindrance, and not by allosteric changes (49). Residues involved in intersubunit interactions and predicted to be affected by the ADP-ribosylation include Glu195, Ser199, Phe200, and Val201. Recently, the Oda model (50) has replaced the Holmes model of filamentous actin. Inspection of the current Oda model reveals few differences in the interaction of these residues with Arg177 of an adjacent subunit. Using this new model, Val194 is also predicted to be involved in subunit interactions with Arg177, and all such residues are high-

![FIGURE 5. Structural model and localization of Photox.](image-url)
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lighted in Fig. 5C. We suggest that the change in proposed actin structure should not present large changes for studies of actin-targeting mARTs.

Photox varies considerably from SpvB in domain organization. Unlike the single-domain SpvB, Photox includes an N-terminal domain from residue 1 to approximately 185. The Photox N-terminal domain is presently an enigma, as it is predicted to be disordered by GeneSilico (26). A BLAST search did not identify any close homologs to the B component of other actin-targeting mARTs indicating that Photox may not function in the typical binary fashion. Based on the disorder, it is predicted that this domain may participate in chaperone binding in a secretion system, or perhaps entry of the toxin into the host cell, but details are not yet known. Intriguingly, genetic neighborhood and gene fusion evidence collected on the STRING database suggests interactions between plu0822 (encoding Photox) and plu0826 (encoding a VgrG-type protein). VgrG may penetrate target cells during type VI secretion to serve as a translocator or an effector (51), presenting the possibility that Photox may infect cells via type VI secretion.

In summary, actin-targeting ADP-ribosyltransferase enzymes have been studied since C. botulinum C2 toxin was first reported in 1986 (23). The most recent addition to this group of toxins was made when SpyA from Streptococcus pyogenes was introduced in 2004 (18). Improved in silico search strategies including limited consensus primary sequence identification, fold-prediction, and secondary structure prediction have allowed for more accurate detection of new putative members of this family. Here we present convincing evidence that Photox is the newest addition to these actin-targeting toxins. We show that Photox shares high homology in the catalytic domain with several other known toxins, but has a domain organization that Photox may infect cells via type VI secretion.
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