Substrate $N^2$ atom recognition mechanism in pierisin family DNA-targeting, guanine-specific ADP-ribosyltransferase ScARP

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ScARP from the bacterium Streptomyces coelicolor belongs to the pierisin family of DNA-targeting ADP-ribosyltransferases (ARTs). These enzymes ADP-ribosylate the $N^2$ amino groups of guanine residues in DNA to yield $N^2$-(ADP-ribos-1-yl)-2′-deoxyguanosine. Although the structures of pierisin-1 and ScARP were revealed recently, the substrate recognition mechanisms remain poorly understood because of the lack of a substrate-binding structure. Here, we report the apo structure of ScARP and of ScARP bound to NADH and its GDP substrate at 1.50 and 1.57 Å resolutions, respectively. The bound structure revealed that the guanine of GDP is trapped between N-ribose of NADH and Trp-159. Interestingly, $N^2$ and $N^3$ of guanine formed hydrogen bonds with the OE1 and NE2 atoms of Gln-162, respectively. We directly observed that the ADP-ribosylating toxin turn-turn (ARTT)-loop, including Trp-159 and Gln-162, plays a key role in the specificity of DNA-targeting, guanine-specific ARTs as well as protein-targeting ARTs such as the C3 exoenzyme. We propose that the ARTT-loop recognition is a common substrate-recognition mechanism in the pierisin family. Furthermore, this complex structure sheds light on similarities and differences among two subclasses that are distinguished by conserved structural motifs: H-Y-E in the ARTD subfamily and R-S-E in the ARTC subfamily. The spatial arrangements of the electrophile and nucleophile were the same, providing the first evidence for a common reaction mechanism in these ARTs. ARTC (including ScARP) uses the ARTT-loop for substrate recognition, whereas ARTD (represented by Arr) uses the C-terminal helix instead of the ARTT-loop. These observations could help inform efforts to improve ART inhibitors.

ADP-ribosylation is an important post-translational modification observed in all living organisms. Many bacterial mono-ADP-ribosyltransferases (ARTs) are known to attach the ADP-ribosyl moiety to specific target proteins and residues (1, 2). The cholera toxin ADP-ribosylates arginine residues in G proteins (3), whereas the pertussis toxin ADP-ribosylates a cysteine residue (4). Diphtheria toxin and the Pseudomonas aeruginosa exotoxin ADP-ribosylate diphthamide, a modified histidine in elongation factor 2 (5, 6). Furthermore, Clostridium botulinum C3 exoenzyme ADP-ribosylates Asn-41 of RhoA (7, 8) and Clostridium perfringens iota toxin A subunit (Ia) ADP-ribosylates Arg-177 of actin (9, 10). These ARTs fall into two major subclasses that are distinguished by conserved structural motifs: H-Y-E in the ARTD subfamily (related to Diptheria toxin) and R-S-E in the ARTC subfamily (related to cholera toxin and clathridial toxins (C3 and Ia)). Traditionally, ADP-ribosylation has been considered a protein modification. However, emerging evidence suggests that DNA ADP-ribosylation is also common. The first DNA-targeting ART was found in pierid butterflies and was thus named pierisin (11, 12). Pierisin ADP-ribosylates calf thymus DNA containing dG–dC and $N^2$ amino group of the guanine residue in DNA to yield $N^2$-(ADP-ribos-1-yl)-2′-deoxyguanosine (Fig. 1a) (13, 14). In contrast, the SCO5461 protein (ScARP) from Streptomyces coelicolor was reported to identify an ART that mainly targets mononucleotides and nucleosides and shares 30% homology with pierisin. ScARP ADP-ribosylates deoxyguanosine (dGuo), GMP, dGMP, and cGMP rather than dsDNA (15), whereas pierisin-1 shows weak ADP-ribosylation activity on dGuo. To date, six pierisins (pierisin-1, -1b, and -2–5), ScARP, and Scabin are considered to belong to the pierisin family (15) and are members of the ARTC subfamily. CARP-1, which is present in certain kinds of edible clams, also ADP-ribosylates calf thymus DNA to produce $N^2$-(ADP-ribos-1-yl)-2′-deoxyguanosine (16, 17). However, CARP-1 and pierisins share very little sequence homology, suggesting that they are not derived from a common ancestral gene (16). Other types of DNA-targeting ARTs have also been identified recently. DarT is found as the ADP-ribosyltransferase of thymines in ssDNA (18). Eukaryotic enzyme poly(ADP-ribose) polymerases (PARPs) can mono-ADP-ribosylate dsDNA ends, which can be reversed by several known ADP-ribosylhydrolases (19–21). This study is the first report of the detailed substrate recognition and ADP-ribosylation.

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This article contains Figs. S1–S5, Table S1 and supporting Refs. 1–2. The atomic coordinates and structure factors (codes 5ZJ4 and 5ZJ5) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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structures guarantee guanine specificity and exclude adenine binding. Notably, Trp-132 on PN-loop is also very important for substrate preference as described under “Discussion.” In pierisin, RhoA comprising Val-43, Ala-56, and Trp-58. This interaction facilitates ADP-ribosylation of the specific asparagine of RhoA (25). However, the target residue recognition mechanism of ARTs via the ARTT-loop is still controversial because of the lack of substrate complex structures. Here, we used X-ray crystallography to reveal the ScARP-GDP (substrate)–NADH complex. It shows a common substrate recognition mechanism of pierisin family enzyme and the first direct observation that the ARTT-loop plays a key role in the specificity of DNA-targeting, guanine-specific ART as well as protein-targeting ART. This study also provides the first complete complex structure of small-molecule targeting ART with minimum elements of ADP-ribosylation. Compared with the Arr structure, which is a rifamycin ADP-ribosyltransferase belonging to ARTD, we propose the general significant substrate recognition differences between ARTC and ARTD.

Results

Structures of apo-ScARP and ScARP-GDP (substrate)–NADH

We unraveled the structure of apo-ScARP and ScARP-GDP (substrate)–NADH at 1.50 and 1.57 Å resolutions, respectively (Fig. 2 and Table S1). The apo structure was solved by molecular replacement using another pierisin family protein, Scabin coordinate (PDB code 5daz), which is an ART from Streptomyces scabies (a plant pathogen that causes scab in potatoes). Structural comparison studies revealed similarities between Scabin, pierisin-1, mosquitocidal toxin, and Community-acquired Respiratory Distress Syndrome Toxin (CARDS TX) with RMSD values of 0.4 Å (159 aa), 2.1 Å (146 aa), 2.2 Å (145 aa), and 1.9 Å (127 aa), respectively (28). It also showed less but definite structural similarity to C3 exoenzyme with an RMSD value of 3.7 Å (88 aa). The key features of the ARTC group (R-S-E motif), including cholera toxin, C3, and Ia are conserved in ScARP (Arg-81–Ser-121–Glu-164) and pierisin (Fig. S1) (29). The STS (121–123) motif is just behind the nicotinamide in Fig. 2a. The amide group of nicotinamide is anchored by the Ser-82 backbone carbonyl and amine. The diphosphate of NADH interacts with Arg-81 and Lys-98. Adenine of NADH is retained by cation–π interaction with Arg-85. The unique features of two disulfide bridges (Cys-46-Cys-76, Cys-180–Cys-194) were also conserved as well as those in Scabin (Fig. S2). In the co-crystal structure with two molecules per asymmetric unit, the electron density of NADH and GDP was clearly visible in the A-molecule, but only NADH density was visible in the B-molecule (Fig. 2b). Thus, we described the active site of the A-molecule with NADH and GDP, NAD+ and NADH bind to ART in a similar conformation, but NADH cannot be a substrate to supply the ADP-ribosyl moiety, and thus it works as an inhibitor. The bent conformation of NADH in ScARP was similar to that in other ARTs (30). The GDP (substrate)-bound structure showed that the guanine ring is stacked between the N-ribose of NADH and Trp-159 (two conformations of the indole ring of Trp-159 can be seen, but both of them are parallel to the purine ring of guanine). The O6 and N1 atoms of guanine are fixed by the main-chain amine of Asn-114 (2.8 Å) and the main-chain carbonyl of Val-111 (2.8 Å), respectively. More importantly, the N2 (NH2) and N3 atoms of guanine form hydrogen bonds with OE1 and NE2 of Gln-162, respectively (Fig. 2, c and d). This provides the N2 atom (NH2) of guanine as the acceptor of the ADP-ribosyl moiety with 4.0 Å from the N1 position of N-ribose. In other words, these binding features guarantee guanine specificity and exclude adenine binding. Notably, Trp-132 on PN-loop is also very important for GDP binding because the PN-loop (Trp-132–Tyr-133–Lys-134–Ser-135–Gly-136) shows the most drastic conformational change upon GDP binding (Fig. 2e). As ribose in GDP (or 2-deoxyribose of DNA) pushes Trp-132, it induces a directional change outward in other PN-loop residues Tyr-133 and Lys-134.

Structural comparisons with pierisin and Scabin

There is a large structural difference between pierisin and ScARP, in the PN-loop (Fig. S3), suggesting a difference in substrate preference as described under “Discussion.” In pierisin,
the long PN-loop and basic cleft were shown to be important for dsDNA by a mutational study (31). Scabin structures were first revealed in apo- and inhibitor-bound forms (32) and very recently in the NADH-bound form (33). A dsDNA (substrate)-bound model was proposed based on the NADH-bound structure. The present GDP–NADH-bound structure contradicts this model as follows: 1) OE1 and NE2 of Gln-162 (QXE of ARTT-loop) form hydrogen bonds with N2 and N3 of guanine (not N2 and N1). 2) The side chain of Trp-132 moves from the apo- to the GDP-bound state (RMSD 4.1 Å) to accept guanosine ribose (Fig. 2, d and e). In other words, the role of Trp-132 seems to be like an adjusting device to accept ribose from guanosine. However, it was suggested that the same tryptophan interacts with other bases in the model of Scabin.

**Assay of ADP-ribosylation of GDP using HPLC**

To reveal the role of Gln-162 and Trp-159 on the ARTT-loop, we measured the ADP-ribosylation activity of WT ScARP and the mutants. Single residue mutations of Gln-162 and Trp-159 (Q162E, Q162N, Q162S, and W159A) affected guanine specificity, leading to decreased ADP-ribosylation activity (Fig. 3). Both crystallographic studies and ADP-ribosylation assay revealed the first direct observation that the ARTT-loop is important for specificity in the DNA-targeting guanine-specific ART.
ACCELERATED COMMUNICATION: Guo recognition by DNA-targeting ART

Structural comparison with C3–RhoA complex and ADP-ribosylation reaction mechanism of ScARP

In the case of C3 exoenzyme, the first turn aromatic residue on the ARTT-loop anchors RhoA, and the second turn Gln on the ARTT-loop forms hydrogen bonds with Asn–41 of RhoA, which is the acceptor of ADP-ribose. Surprisingly, the relative positions of NAD(H), the acceptor of ADP-ribose, and Gln of the ARTT-loop are exactly the same position in two different complex structures of C3 and ScARP (Fig. 4). These relative position similarities using the ARTT-loop were conserved between protein-targeting ART and DNA-targeting ART, suggesting their importance for ADP-ribosylation. In guanine-specific ScARP, the reactions are summarized as follows. 1) Trp-159 on the first turn of ARTT-loop and Gln-162 on the second turn of ARTT-loop place the guanine close to the NC1 position of N-ribose, cooperatively. 2) The second turn Glu-164 of the ARTT-loop is important for cleaving NAD⁺ to produce nicotinamide and the oxocarbenium cation. 3) The oxocarbenium cation is transferred to N² of guanine and produces N²-(ADP-ribose-1-yl)-2’-deoxyguanosine. It should be noted that the role of Gln-162 is not only to grip N² of guanine but also to direct the orbital of the lone pair of N² against NC1. In general, it is reported that an Sₐ₁ reaction occurs via the oxocarbenium cation in ART (34). The short distance (4.0 Å) between NC1 of N-ribose (electrophile) and the N² atom of guanine (nucleophile) may permit an Sₐ₁ reaction with a direct back attack using the N² atom as a nucleophile. In the case of la-actin, the Sₐ₁ strain-alleviation model was suggested as a possible reaction mechanism based on its complex crystal structure (24, 35). It was reported that ScARP produces only a single isomer of N²-(ADP-ribose-1-yl)-2’-deoxyguanosine and that it anomerized within 4 h. However, which isomer acted as the initial product was an open question (15). This study suggests that the initial product is in the α form, which is then converted to the β form by nonenzymatic anomerization.

Discussion

Although the overall structures among pierisin family members are similar, their substrate preferences are different. However, the present complex structure provides an important insight into the common guanine specificity in the pierisin family. Recently, the structure of pierisin-1 was revealed (31). In pierisin-1, the key residues (Trp-160, Gln-163, and Glu-165 on the ARTT-loop) are also conserved. Pierisin seems to have a preference for dsDNA, but its final product, N²-(ADP-ribose-1-yl)-2’-deoxyguanosine, is the same as that of ScARP. Scabin was reported as a DNA-targeting mono-ART but shows preference for dsDNA with a single-base overhang rather than a blunt end, with the same guanine specificity (as there is no activity with dl, it was proposed that the product is the same as that of ScARP and pierisin) (33). There might be some varieties with a greater preference for dsDNA, ssDNA, and mononucleotide in each of the pierisin family enzymes. These differences in substrate preference are mainly due to the differences in the PN-loop and its nearby sites in ScARP, Scabin, and pierisin. The electrostatic potentials show large differences between pierisin-1 and Scabin/ScARP (Fig. S2). In pierisin-1, the basic surface is created by the basic residues in the PN-loop (Lys-122, Lys-123, Lys-124, Arg-130, and Arg-134), Arg-67, Arg-181, Arg-187, and Arg-211. In particular, Lys-122, Lys-123, Lys-124, Arg-181, and Arg-187 are indispensable for DNA binding of pierisin-1 (31). These residues are not conserved in ScARP and Scabin, and there are no obvious basic regions in ScARP. Scabin has a small basic region created by Lys-130 in the PN-loop, Lys-180, Arg-183, and Lys-186. It was proposed that the basic region was associated with dsDNA binding (36). Although the substrate preferences are different among the pierisin family, the ARTT-loop that is essential for binding with GDP is conserved among the three enzymes. This means that the described key guanine recognition via the ARTT-loop and ADP-ribosylation mechanism is common in DNA-targeting guanine-specific ARTs. On the basis of this idea, we considered the model structure of pierisin-1 with dsDNA. We built the model of pierisin-1 with dsDNA containing flipped O⁶-methylguanosine (PDB code 1T38, O⁶-alkylguanine-DNA alkyltransferase (AGT)) (Fig. S4). We used dsDNA containing a flipped guanosine because guanosine cannot bind with the ARTT-loop unless it is flipped from the base stack, and we superimposed the flipped O⁶-methylguanosine moiety on the GDP overlaid on pierisin. Although dsDNA partially overlaps with pierisin-1, this rough model gave three important insights. 1) The basic cleft of pierisin-1 is used for binding with dsDNA. 2) Guanosine of dsDNA has to be flipped from the base stack to bind with the ARTT-loop. 3) Arg-130 in PN-loop protrudes into the DNA duplex to promote flipping guanosine out of the base stack in the same way as Arg-128 of AGT (PDB code 1T38) (37).

There is another report of small molecule-targeting ART: ADP-ribosylation of antibiotic rifamycin results in antibiotic resistance. The structure of the enzyme Arr from Mycobacterium smegmatis was reported in the rifampin (a semisynthetic derivative of the natural product of rifamycin B)-bound state (38), but ADP-ribosylation mechanism was unclear because of no NAD(H)-bound structure. Arr belongs to the ARTD sub-class, which has the H-Y-E motif instead of the R-S-E motif found in ARTC and lacks the ARTT-loop. In another ARTD–substrate complex structure ExoA–eEF2–NAD⁺, the ADP-ribosylation mechanism was also unclear because the structure exhibited a long distance between electrophile and nucleophile (23, 39). In this case, the conformational change of nucleophile...
(diphthamide) during the reaction was speculated. The structural comparison with ScARP–GDP–NADH clearly showed the same spatial arrangements of NC1 of N-ribose (electrophile) close to the acceptor oxygen atom of rifampin (nucleophile) in the model of Arr–rifampin–NAD(H) (Fig. 5), providing the first clear insight of ADP-ribosylation mechanism in ARTD. In ARTD, rifampin (substrate) was recognized by the C-terminal H9251α-helix instead of the ARTT-loop in ARTC. By structure-based phylogenetic analysis, it was confirmed that two small molecule-targeting ARTs, ScARP and Arr, localize in ARTC and ARTD, respectively (Fig. S5). Comparing complete coordinates of a small molecule targeting ART’s with minimum elements of ADP-ribosylation, we have identified the substrate recognition difference between ARTD and ARTC for the first time. Our study is not limited to the bacterial ARTCs. This leads to the insights of ARTD, including mammalian PARP. Furthermore, these deeper understandings of substrate recognition of both ARTC and ARTD would lead to the development of better inhibitors of ARTC and ARTD.

**Experimental procedures**

**Expression and purification of ScARP**

The ScARP gene (UniProt ID: Q9L1E4), sco5461, without the signal peptide (residues 43–204) was cloned into a pRhA plasmid with N-terminal hexahistidine and SUMO protein and was overexpressed in the *Escherichia coli* BL21 Star (DE3) strain. The transformants were cultivated in LB medium containing 50...
Crystallization

Crystallization was carried out using the hanging drop vapor diffusion method. Single crystals of apo-ScARP were obtained by two-step crystallization. In the first crystallization step, the drop was composed of equal volumes of 20 mg/ml ScARP and a reservoir solution containing 0.1 M HEPES, pH 7.0, and 8% PEG8000. Bad quality crystals appeared within a few days at 20 °C. These crystals were crushed, diluted with the reservoir solution, and used for the second crystallization step as a seed. In the second crystallization step, the drop was composed of 1 μl of 10 mg/ml ScARP, 1 μl of the reservoir solution containing 0.1 M sodium acetate, pH 5.0, and 15% 2-methyl-2,4-pentanediol, and 0.1 μl of the diluted seed solution. This condition was determined by microseed matrix screening (40). The second crystallization generated single crystals at 20 °C. Prior to data collection, the crystals were briefly soaked in the second crystallization reservoir solution, including 20% xylitol. To obtain the ScARP–GDP–NADH complex crystals, a solution containing 17 mg/ml (0.9 mM) ScARP, 9 mM GDP, and 9 mM NADH was incubated for 1 h at 4 °C before crystallization. The drop comprised equal volumes of the protein solution and the reservoir solution containing 0.1 M ammonium acetate, pH 5.0, and 7% PEG10000. Rod-shaped crystals appeared within a few days at 4 °C. Prior to data collection, the crystals were transferred to a mixture of paraffin and paratone oils (Hampton).

Data collection and structure determination

Diffraction data sets were collected at 100 K on a beamline PF BL-5A. The structure of ScARP was solved through molecular replacement using the program PHASE (41) using the Scabin structure (Protein Data Bank code 5DAZ). The model was then iteratively built using COOT (42) and refined using phenix. refine (43). The atomic model and structure factors were deposited at Protein Data Bank under accession code 5ZJ4 (apo-ScARP) and 5ZJ5 (ScARP–GDP–NADH).

Activity measurement

To measure the ADP-ribosylation activity, WT ScARP and the mutants were prepared as follows. The ScARP gene (Uni-Prot ID: Q9L1E4) without the signal peptide (residues 43–204) was cloned into a pET15b plasmid with N-terminal hexahistidine and was overexpressed in the E. coli BL21(DE3) strain. The transformants were cultivated in LB medium containing 50 μg/ml ampicillin at 37 °C until the absorbance at 620 nm reached 0.6. After inducing expression with 0.5 mM isopropyl β-D-1-thiogalactopyranoside, the culture was incubated for 16 h at 16 °C. The harvested cells were resuspended in lysis buffer containing 50 mM Tris, pH 8.0, 300 mM NaCl, and 20 mM imidazole, disrupted by sonication, and centrifuged. The supernatant was loaded onto a Ni-NTA–agarose column. After washing the column with lysis buffer, ScARP was eluted with 50 mM Tris, pH 8.0, 300 mM NaCl, and 400 mM imidazole. The ScARP fractions were finally loaded onto a Superdex 75 column (GE Healthcare) with 10 mM Tris, pH 8.0, and 100 mM NaCl, concentrated to 1.5 mg/ml, rapidly frozen in liquid nitrogen, and stored at −80 °C.

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