Structure of human ADP-ribosyl-acceptor hydrolase 3 bound to ADP-ribose reveals a conformational switch that enables specific substrate recognition

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ADP-ribosyl-acceptor hydrolase 3 (ARH3) plays important roles in regulation of poly(ADP-ribosylation), a reversible post-translational modification, and in maintenance of genomic integrity. ARH3 degrades poly(ADP-ribose) to protect cells from poly(ADP-ribose)–dependent cell death, reverses serine mono(ADP-ribosylation), and hydrolyzes O-acetyl-ADP-ribose, a product of Sirtuin-catalyzed histone deacetylation. ARH3 preferentially hydrolyzes O-linkages attached to the anumeric C1 of ADP-ribose; however, how ARH3 specifically recognizes and cleaves structurally diverse substrates remains unknown. Here, structures of full-length human ARH3 bound to ADP-ribose and Mg2++, coupled with computational modelling, reveal a dramatic conformational switch from closed to open states that enables specific substrate recognition. The glutamate flap, which blocks substrate entrance to Mg2+ in the unliganded closed state, is ejected from the active site when substrate is bound. This closed-to-open transition significantly widens the substrate-binding channel and precisely positions the scissile 1′-O-linkage for cleavage while securing tightly 2′- and 3′-hydroxyls of ADP-ribose. Our collective data uncover an unprecedented structural plasticity of ARH3 that supports its specificity for the 1′-O-linkage in substrates and Mg2+-dependent catalysis.

Regulation of poly(ADP-ribosylation) (PARylation), a reversible post-translational modification (PTM) of proteins, is required for maintaining genomic integrity and cellular responses to DNA damage (1, 2). The addition of poly(ADP-ribose) (PAR) to proteins by PAR polymerase 1 (PARP1; also known as ARTD1) plays a pivotal role in the repair of DNA single- and double-strand breaks (3–6). However, excessive PARylation by PARP1 often interferes with protein function and may activate parthanatos, a PAR-dependent cell death pathway, which involves the translocation of PAR to the cytoplasm, eventually triggering the PAR-dependent release of apoptosis-inducing factor (AIF) from mitochondria (7, 8). AIF released from mitochondria then goes to the nucleus, leading to DNA cleavage and cell death. The cellular level of PARylation and PAR is therefore tightly controlled by PAR synthesis and turnover.

In mammals, two enzymes, ADP-ribosyl-acceptor hydrolase 3 (ARH3; also known as ADPRHL2) and PAR glycohydrolase (PARG), function in tandem to reverse PARylation (9). These hydrolytic enzymes commonly cleave the α(1′-2′) O-glycosidic linkages in PAR chains (Fig. 1a) (10, 11). PARG has both exo- and endoglycohydrolase activities, acting at terminal and internal sites of PAR chains, respectively (9, 12–14). Thus, in addition to free ADP-ribose (ADPR), PARG generates short chains of PAR that serve as a potent cell death signal (9). In contrast, ARH3 appears to catalyze primarily exocytic cleavage of PAR, generating free ADPR (9). Consistent with this difference in biochemical activity, ARH3 protects cells from oxidative stress–induced parthanatos by lowering the cytoplasmic PAR level (9). ARH3 knockout cells are healthy under unstressed conditions. However, following H2O2-induced DNA damage, these cells show accumulation of cellular PAR, in particular in the cytoplasm where it interacts with mitochondria, leading to AIF cleavage and release, enhanced nuclear accumulation of AIF, and overall increased activity of the parthanatos pathway (9). Taken together, these findings indicate that ARH3 is a key enzyme that not only controls PAR content but also determines cell fate during the DNA damage response.

PARG is unable to reverse a protein-bound mono(ADP-ribosylation) (MARylation), the last step in completely erasing poly(ADP-ribosylation) (15). This MARylation mark, particularly on acidic residues, is removed by macrodomain-containing proteins with MAI hydrolytic activity such as terminal ADP-HPD length; r.m.s.d., root mean square deviation; ADP-HPD, adenosine diphosphate (hydroxymethyl)pyrrolidine-2′,3′-diol.
ribose protein glycohydrolase (TARG1), MacroD1, and MacroD2 (16–18). It has been shown that serine-specific ADP-ribosylation, a PTM specifically generated by PARP1/HPF1 and PARP2/HPF1 complexes, is the major cellular PTM following DNA damage (19–21). Importantly, in addition to PAR degrada-
tion, ARH3 was identified as the cellular eraser of serine MARylation marks (20, 22). Furthermore, ARH3 can specifically digest O-acetyl-ADPR, a product of Sirtuin-catalyzed NAD\(^+\)-dependent histone deacetylase reactions, which regulate diverse biological processes, including chromatin remodel-
ing (11, 23, 24). Therefore, ARH3 is the only known enzyme that can specifically hydrolyze PAR, MAR PTMs, and O-acetyl-
ADPR. For all these substrates, ARH3 preferentially hydrolyzes the scissile \(1\)-O-linkage attached to the anomeric C1\(^\text{\text{o}}\) position of ADPR (24) (Fig. 1a). However, the structural basis for the specificity of ARH3 for the 1\(^{-}\)-O-linkage is unknown. Collectively, ARH3 is a distinctive, multitasking enzyme that controls two biologically important \(1\)-O-dependent cellular signaling pathways.

The structure of the unliganded human ARH3, lacking the N-terminal 16 residues (ARH3\(^{\text{N16}}\)), shows a compact all-\(\alpha\)-helical fold with a binuclear Mg\(^{2+}\) center, which constitutes an archetype of the ARH superfamily (25). This di-Mg\(^{2+}\)–
containing catalytic center (Mg\(^{A}\) and Mg\(^{B}\)) is not found in PARG and is consistent with the Mg\(^{2+}\)-dependent ADP-ribo-
syl-acceptor hydrolase activity of ARH3 (11, 23). Consistently, mutations of Mg\(^{2+}\)-coordinating residues of ARH3 led to a drastic decrease in PAR- and MAR-acceptor–hydrolyzing activities (22, 25). However, we lack a fundamental mechanistic understanding of how ARH3 specifically recognizes and cleaves structurally diverse substrates in a Mg\(^{2+}\)-dependent manner.

To better understand the molecular mechanism of ARH3 activities, we determined high-resolution crystal structures of full-length human ARH3 bound to Mg\(^{2+}\) and ADPR, a product and an effective inhibitor of ARH3 activities (23, 24). Coupled with computational analysis of ARH3–PAR substrate interac-
tions, these structures reveal that substrate binding drives a large-scale conformational transition from an unliganded closed state to an open incision–competent enzyme state. Glu\(^{41}\) from the “glutamate flap,” which blocks the entry of substrate in the closed state, is completely ejected from the binuclear Mg\(^{2+}\) center. The concomitant restructuring of the active site uncovers the catalytically important Mg\(^{B}\), widens the leaving group–binding channel, and precisely positions the scissile 1\(^{-}\)-O-linkage for hydrolysis while tightly securing the 2\(^{-}\)- and 3\(^{-}\)-OH groups of ADPR. Unique structural features and conformational flexibility of ARH3 strongly support ARH3’s specificity for the 1\(^{-}\)-O-linkage in structurally diverse substrates and Mg\(^{2+}\)-dependent catalysis.

Results

Structure of human ARH3\(^{\text{FL}}\)–ADP-ribose–Mg\(^{2+}\) complex

The crystal structure of apo-ARH3\(^{\text{N16}}\) explained the lack of ADPR binding (25) (Fig. S1). We reasoned that the full-length ARH3 (ARH3\(^{\text{FL}}\)) might provide opportunities to capture ARH3 in an ADPR-bound active form. The purified ARH3\(^{\text{FL}}\) is likely metal-free given its comparable basal activity without the addition of Mg\(^{2+}\) and in the presence of EDTA (Fig. 1b). The addi-
tion of Mg\(^{2+}\) markedly enhances ARH3\(^{\text{FL}}\)-mediated PAR hydrolysis (Fig. 1b), which is consistent with a previous report (11). ARH3\(^{\text{FL}}\) shows maximal activity with Mg\(^{2+}\) followed by Mn\(^{2+}\) and Ca\(^{2+}\) (Fig. 1b). Unlike DraG, the closest functional homolog that has a strong preference for Mn\(^{2+}\) over Mg\(^{2+}\) (25–27), ARH3 exhibits rather comparable activity with either Mg\(^{2+}\) or Mn\(^{2+}\). We crystallized ARH3\(^{\text{FL}}\) in complex with ADPR and Mg\(^{2+}\) and determined its crystal structure at 1.7-Å resolution (Fig. 1d). Thus, the numbering of amino acid residues in this report deviates by 16 from that used for the apo-
ARH3\(^{\text{N16}}\) structure (25), but it is consistent with those used by Oka et al. (11) and Apblanjal et al. (22). ARH3\(^{\text{FL}}\) binds to one ADPR as evidenced by a clear and strong difference electron density at the active site (Fig. 1c).

ARH3\(^{\text{FL}}\) adopts a compact all-\(\alpha\)-helical fold with a central deep ADPR-binding cleft, a signature of the ARH3 superfamily (Figs. 1 and 2). The ARH3–ADPR–Mg\(^{2+}\) complex structure identifies two unique structural elements of ARH3, the “ade-
nine cap” and glutamate flap, which undergo a structural rearrangement upon ADPR binding and strongly contribute to the specific substrate recognition of ARH3 (Figs. 1d and 2). The binuclear Mg\(^{2+}\) catalytic center lies at the heart of a long, J-shaped substrate-binding channel. This structural feature enables ARH3 to bind and align both ADPR and the leaving group, e.g., two ADPR units (n ADPR and the n – 1 ADPR leaving group) in PAR substrates, for specific cleavage (Fig. 1a and d).

The N-terminal 13 amino acids in the N-terminal extension are disordered, which is consistent with their dispensable role in ARH3 function (25). However, unexpectedly, Arg\(^{18}\) in the N-terminal extension, previously replaced by alanine in the apo-ARH3\(^{\text{N16}}\) structure (25), contributes to ARH3 folding. Arg\(^{18}\) makes helix-capping interactions with the main-chain carbonyls of Ser\(^{161}\) and Leu\(^{162}\) in \(c7\) to stabilize \(c7\) (Fig. S2). Arg\(^{18}\) further stabilizes ARH3 folding by forming a hydrogen bond with the side-chain carbonyl of Gln\(^{361}\) of \(\alpha19\) and a van der Waals contact with the side chain of Phe\(^{23}\) of \(\alpha1\). However, given the fully functional PAR hydrolysis activity of ARH3\(^{\text{N16}}\) (25) and a remarkably long distance between Arg\(^{18}\) and Glu\(^{41}\) (~32 Å), it is unlikely that Arg\(^{18}\) contributes to the observed conformational changes.

Structural comparison of the unliganded and ADPR-bound forms of ARH3 reveals dramatic conformational changes in the Glu\(^{41}\)-containing flap motif, which we named the glutamate flap (Glu\(^{41}\)-flap) (r.m.s.d. of 2.5 Å, comparing 52 C\(^{\text{\text{o}}}\) atoms in the Glu\(^{41}\)-flap and its flanking residues of the unliganded ARH3\(^{\text{N16}}\) and complex D of the ARH3–ADPR–Mg\(^{2+}\) complex) (Fig. 1d). The Glu\(^{41}\)-flap is composed of the end of \(c\) that exists as a 3\(_{10}\)-helix in the unliganded ARH3, \(c2\), and a flexible loop connecting \(c1\) and \(c2\) (L1) (Figs. 1d and 2). This substrate-
induced conformational transition fully exposes the bimetallic catalytic center for substrate engagement and to allow efficient hydrolysis to occur as described below. Together, these findings imply that ARH3 can exist in at least two states: a substrate-bound “open” state and an unliganded “closed” state.

Four ARH3–ADPR–Mg\(^{2+}\) complexes were found in the asymmetric unit (Fig. S3). The r.m.s.d. difference between the structures of four ARH3–ADPR–Mg\(^{2+}\) complexes (complexes
ARH3 specifically exposes 1″-OH of ADP-ribose

ADPR is located at the deep ADPR-binding cleft of ARH3. All three parts of ADPR (adenosine, diphosphate, and the distal ribose″) make extensive contacts with ARH3 (Figs. 1d and 3). ADPR has a surface area of 694 Å² of which ~80% (555 Å² on average in complexes A–D) is buried by direct contacts with ARH3. Overall, this matrix of ARH3–ADPR–Mg²⁺ interactions specifically exposes 1″-OH, corresponding to the scissile O-linkage in substrates (Figs. 1a and 3c), toward the catalytic Mg⁶⁺, strongly supporting its specificity for the 1″-O-linkage for substrate cleavage.

The distal ribose″ of ADPR lies adjacent to the binuclear Mg²⁺ center where a group of acidic and polar residues extensively contact Mg²⁺ ions and ADPR. The interactions between the ribose″ and two Mg²⁺ ions are asymmetrical with more extensive contacts on Mg⁶⁺. 3″-OH of the ribose″ is directly coordinated by Mg⁶⁺, and it is additionally hydrogen-bonded with the side chain of Asn¹⁵⁻ (Fig. 3, b and c). A water molecule (μ-aqua ligand) that bridges Mg⁶⁺ and Mg⁶⁺ simultaneously engages 2″-OH of the ribose″ with an unusually short distance (2.2 Å). These Mg²⁺-mediated ARH3 interactions with 2″- and 3″-OH appear to secure tightly the ribose″ to facilitate efficient cleavage of substrates at the 1″-O-linkage. In support of this hypothesis, in contrast to 2″- and 3″-OH groups, 1″-OH of ADPR that corresponds to the 1″-O-linkage in substrates is sol-
vent-accessible and exposed to the leaving group–binding site (Figs. 1d and 3c), consistent with cleavage at the C1’ position (11, 20, 24). Another water molecule (W1) is axially liganded to Mg⁶⁺ and makes a hydrogen bond with 1’-OH (Fig. 3c). This W1 ligand is located proximal to the anomeric C1’ and appears well aligned for nucleophilic attack of C1’ during catalysis. At the other side of the binuclear metal center, the carboxyl group of Asp77 is aligned for nucleophilic attack of C1 of Glu41-flap of ARH3 that undergoes a large conformational change upon ADPR binding is indicated by a red box. A part of L2 of DraG (L2 wall) completely blocks the conformational change of α1 and restricts its activity to cleavage of mono(ADP-ribosyl)ated substrates. The end of α1 in ARH3, which exists as 310-helix in the unliganded form and undergoes 310-to-α transition upon ADPR binding (Fig. 4a), is indicated by a blue bar. Two aromatic residues in DraG that stabilize the L2 wall are indicated by a red triangle. Asp⁷⁷ in DraG that is essential for the cleavage of MARylated arginine is indicated by a blue triangle.

Despite the structural similarity between ARH3 superfamily members, the binding orientation of ADPR in ARH3 is remarkably different from that in DraG (28) (r.m.s.d. of 2.6 Å, comparing all Ca positions of complex D and the DraG–ADPR complex (Protein Data Bank code 2WOE)) (Figs. 3a and S4a). This distinct mode of ADPR binding can be accounted for by a unique adenine-binding module of ARH3, the adenine cap, which consists of α6, α12, and a loop flanking the C terminus of α6 (L6). Notably, α6 and α12 are missing in DraG (Figs. 2 and 3a). Two aromatic amino acids from the adenine cap, Phe¹⁴³ and Tyr¹⁴⁹, sandwich the adenine base through extensive π–π stacking interactions (Fig. 3a). In line with this finding, the ARH3-Y149A mutant showed a drastic decrease in PAR- and MAR-acceptor–hydrolyzing activities (22, 25). Leu²³⁵ from the flexible α12 in the adenine cap moved ~2.7 Å toward the adenine ring relative to the unliganded ARH3, forming a van der Waals contact with N6 of the adenine base (Fig. 3, a and b). The chemical selectivity of the adenine base is further enhanced by hydrogen bonds between the backbone carbonyl of Gly¹⁹⁷ and N6 and between the backbone nitrogen of Tyr¹⁴⁹ and N7 (Fig. 3, a and b). The diphosphate moiety interacts with the side chains of Ser¹⁴⁸ and His¹⁸² and the main-chain nitrogen of Gly¹¹⁹. Consistently, substitution of Ser¹⁴⁸ and His¹⁸² with alanine led to loss of ADP-ribosyl-acceptor hydrolase activities (22, 25).

A conformational switch of ARH3 enables specific substrate recognition

The most striking feature in the ARH3–ADPR–Mg²⁺ complex is the dramatic conformational change of the Glu⁴¹-flap (Fig. 4). The end of α1, which is at the beginning of the Glu⁴¹-
flap, exists as a kinked $3_{10}$-helix structure in the unliganded ARH3 (Fig. 4a). ADPR binding drives a $3_{10}$-to-$a$ transition, leading to $\sim 27^\circ$ of rotation from the helical axis of the $3_{10}$-helix. This rotation induces a concomitant $\sim 8.5$-Å displacement of L1 away from the active site, which accompanies $\sim 24^\circ$ of rotation in $\alpha 2$ (Fig. 4a). Consequently, ADPR binding results in an $\sim 4.5$-Å movement of the carboxylate of Glu$^{41}$ of the Glu$^{41}$-flap away from the catalytic Mg$^2+$. The straightened conformation of $\alpha 1$ is stabilized by new intrahelical stacking interactions within $\alpha 1$ among residues Phe$^{39}$, Tyr$^{40}$, and His$^{63}$ (Fig. 4a).

Mechanistically, the observed conformational switch of the Glu$^{41}$-flap is required for specific substrate recognition. In the unliganded state, Glu$^{41}$ of the Glu$^{41}$-flap completely masks Mg$^B$ from access to substrate (Fig. 4c). This closed state is therefore enzymatically inactive, and Glu$^{41}$ must be ejected away from Mg$^B$ to allow substrate to enter the dimetallic catalytic center. In support of this hypothesis, the ARH3–ADPR–Mg$^{2+}$ complex shows a conformational switch in the Glu$^{41}$-flap from a closed state to an open state. This substantial active-site restructuring unmasks Mg$^B$ and significantly widens the leaving group–binding site (Figs. 1d and 4, b and c), which now allows substrate entrance to the dimetallic catalytic center. This open conformation of ARH3 provides an optimal alignment between the scissile 1''-O-linkage and catalytic groups (Mg$^{2+}$ and catalytic residues) and therefore constitutes an “incision-competent” enzyme state (Figs. 3d and 4b). Collectively, the Glu$^{41}$-flap controls substrate access to the binuclear Mg$^{2+}$ center, and its closed-to-open conformational switch enables specific binding and cleavage of substrates.

To gain further mechanistic insights into substrate hydrolysis, we modeled di-ADPR, a substrate with the largest leaving group (Fig. 1a), to the active site of ARH3 in catalytic position (Fig. 4, d and c). In this ARH3–di-ADPR–Mg$^{2+}$ model, the $n - 1$ ADPR leaving group snugly fits into the curved $J$-shaped substrate binding channel (Fig. 4b), whereas $n$ ADPR occupies the identical site as that seen in the ARH3–ADPR–Mg$^{2+}$ com-
plex. Notably, 3’-OH of the adenosine ribose of n − 1 ADPR is directly coordinated to Mg₈, replacing the axially coordinated water ligand (W1), and it is positioned within hydrogen-bonding distance to Asp³¹⁴ and Glu⁴¹ (Figs. 3d and 4b). These findings suggest that Mg₈ is important for catalysis by securing ADPR and the leaving group to promote the subsequent cleavage.

Structural superposition of ADPR-bound forms of ARH3 and DraG reveals that the observed 3₁₀-to-α transition of α₁ and the conformational flexibility of the Glu⁴¹-flap are unique in ARH3. Analysis of secondary structures using Dictionary of Secondary Structure of Proteins (DSSP) (29) indicates that DraG lacks the flexible 3₁₀-helix structure at the end of α₁ (Fig. 2). Furthermore, a part of L₂ of DraG (named the “L₂ wall”) tightly caps α₁ through an edge-stacking π–π interaction between the side chains of Trp⁹⁰ from L₂ and Phe²⁹ from α₁ (Figs. 3a and S4). This structural arrangement of DraG effectively restricts the conformations of α₁ and L₁ (corresponding to the Glu⁴¹-flap in ARH3) to the closed state in which substrate access to the leaving group–binding site is prevented (Fig. 4a). The DraG mechanism instead involves a ring opening of the ribose, which positions the scissile 1”-O-linkage in close proximity to Asp⁹⁷ of DraG for cleavage (28). This structural feature would enforce DraG substrate specificity to MARylated arginine (28). In ARH3, Phe²⁹ and Trp⁹⁰ of DraG are replaced with alanine and serine, respectively, relieving the constraining interaction. Consequently, the L₂ of ARH3, equivalent to the L₂ wall of DraG, is disordered. Furthermore, Asp³⁹ of DraG is replaced with glycine in ARH3 (Fig. 2). Taken together, the conformational flexibility of the Glu⁴¹-flap, the lack of the L₂ wall, metal preference for Mg²⁺, and the lack of the catalytic residue equivalent to DraG Asp⁹⁷ in ARH3 (25) (Fig. 2), all suggest a distinct catalytic mechanism for ARH3 and explain different substrate specificity in ARH3.

**Binuclear metal center of the ARH3–ADPR–Mg²⁺ complex**

In the ARH3–ADPR–Mg²⁺ complex, two Mg²⁺ ions (Mg⁸ and Mg⁹) have an octahedral coordination geometry and are fully occupied with only 3.3-Å intermetal distance (3.8 Å in the unliganded ARH3 structure). They are bridged by the bidentate carboxyl group of Asp³¹⁴ as well as by the μ-aqua ligand (Fig. 3c). The bridging μ-aqua ligand is nearly symmetrically coordinated by two Mg²⁺ ions (1.9 and 1.8 Å to Mg⁸ and Mg⁹, respectively) and fixes 2”-OH of the ribose of ADPR (Fig. 3c).

In contrast to Mg⁸, whose coordination ligands remain identical (including Thr⁷⁶, Asp⁷⁷, Asp⁷⁸, and Asp³¹⁴), the Mg⁹ coordination setup is dynamically changed. In the unliganded ARH3, Glu³¹ of the Glu⁴¹-flap is coordinated by Mg⁹. Upon PAR substrate binding, 3’-OH of the adenosine ribose of the n − 1 ADPR leaving group replaces Glu³¹ and directly coordinates Mg⁹. Thus, in the substrate-bound state, Mg⁹ engages both ADPR and the leaving group to precisely expose the scissile 1”-O-linkage for cleavage (Figs. 3d and 4b). Asp³¹⁴ and Glu³¹ further aid in the correct positioning of the n − 1 ADPR leaving group by interacting with 3’-OH of the adenosine ribose. Finally, in the ARH3–ADPR (product) complex, the leaving group departs, and a new water ligand (W1) is axially coordinated by Mg⁸ (Figs. 3c and 4b). This dynamic switching of the metal-site makeup in each catalytic step of ARH3 is consistent with Mg²⁺-dependent ARH3 activity.

**Asp³¹⁴ is essential for the formation of binuclear metal center**

The structure of the ARH3–ADPR–Mg²⁺ complex shows that the dimetallic catalytic center of ARH3 is tightly packed against ADPR and appears ideally optimized for cleavage of the scissile 1”-O-linkage in substrates. Therefore, a subtle change in the active-site arrangement may result in a detrimental effect on ARH3 functions. Consistently, a conservative substitution of Asp³¹⁴ with glutamate led to the loss of enzymatic activity (22, 25) (Fig. S5a). To gain further insights into this structure–function relationship, we determined the crystal structure of ARH3D³¹⁴E bound to ADPR and Mg²⁺ at 1.6-Å resolution (Figs. 5 and S5b). The overall structure and ADPR-binding mode are nearly identical to those for the ARH3WT–ADPR–Mg²⁺ complex (r.m.s.d. of 0.2 Å, comparing all Ca positions of complexes D of ARH3WT and ARH3D³¹⁴E). Strikingly, however,
Structure of human ARH3 bound to ADP-ribose and Mg\textsuperscript{2+}

Figure 5. Asp314 is essential for the formation of the binuclear metal center. Structural comparison of the WT and catalytically inactive ARH3-D314E mutant reveals a side-chain flipping of Glu314, which leads to the loss of Mg\textsuperscript{B}. This suggests that Asp314 is essential for the formation of the binuclear metal center in ARH3 and that Mg\textsuperscript{B} is required for catalysis.

Mg\textsuperscript{B} is completely missing in the ARH3-D314E-ADPR-Mg\textsuperscript{2+} complex, and the side chain of Glu314 is flipped out from the binuclear metal center, presumably due to a steric clash caused by the one-carbon-longer side chain in Glu314 (Fig. 5). The distance between Glu314 and the corresponding Mg\textsuperscript{B} in the ARH3-WT-ADPR-Mg\textsuperscript{2+} complex is beyond the range of direct coordination (3.7 Å), explaining the loss of Mg\textsuperscript{B} and the loss of enzymatic activity. Consistent with the important role of Mg\textsuperscript{B} in securing ADPR (Fig. 3c), the overall B factors for atoms in the ribose\textsuperscript{O} of ADPR in the ARH3-D314E-ADPR-Mg\textsuperscript{2+} complex are significantly higher than those in the ARH3-WT-ADPR-Mg\textsuperscript{2+} complex. Collectively, these findings indicate that Asp314 is required for the formation of the binuclear Mg\textsuperscript{2+} center and suggest a critical role of Mg\textsuperscript{B} for catalysis.

Discussion

Our structures of the ARH3-ADPR-Mg\textsuperscript{2+} complex and a computational model of the ARH3-di-ADPR-Mg\textsuperscript{2+} complex reveal the previously unknown conformational plasticity of the Glu\textsuperscript{41}-flap, which strongly supports ARH3 specificity for the 1\textsuperscript{-O}-linkage for cleavage. Our studies also explain the published mutational analysis data for ARH3. These structural findings are consistent with Mg\textsuperscript{2+}-dependent hydrolysis and provide important clues to the catalytic mechanism of ARH3. First, the observed conformational switch in ARH3 is required to specifically recognize substrates. In the closed unliganded state, Glu\textsuperscript{41} coordinates Mg\textsuperscript{B} and prevents substrates from entering into the dimetallic catalytic center (Fig. 6). To engage substrates, the Glu\textsuperscript{41}-flap is completely moved away from Mg\textsuperscript{B}, inducing substantial active-site rearrangement from a closed to an open state. This conformational transition now allows substrates to enter the active site of ARH3. Second, our structures strongly support the observation that ARH3 favors the 1\textsuperscript{-O}-linkage in substrates for cleavage (24). In the ARH3-ADPR-Mg\textsuperscript{2+} complex, 2\textsuperscript{-} and 3\textsuperscript{-}OH of ADPR are secured by Mg\textsuperscript{2+} ions, the bridging μ-aqua ligand, and active-site residues, whereas 1\textsuperscript{-}OH corresponding to the scissile 1\textsuperscript{-O}-linkage in substrates is specifically exposed to the open leaving group-binding site (Fig. 3c). Substrates containing 2\textsuperscript{-} or 3\textsuperscript{-}-O-linkage therefore would result in a serious steric clash with the tightly packed binuclear metal center of ARH3, leading to inefficient substrate cleavage. Consistently, IC\textsubscript{50} values for ARH3 inhibition by 2\textsuperscript{-} or 3\textsuperscript{-}-N-acetyl-ADPR, analogs of O-acetyl-ADPR, were significantly higher than that for ADPR (24). In our ARH3-di-ADPR-Mg\textsuperscript{2+} model, the di-ADPR is slightly bent due to the simultaneous engagement of 3\textsuperscript{-}-OH of n ADPR and 3\textsuperscript{-}OH of n - 1 ADPR by Mg\textsuperscript{B} (Figs. 3d and 6). This structural feature would likely further reinforce the correct positioning of the scissile 1\textsuperscript{-O}-linkage for efficient cleavage (Fig. 6). Third, the substrate-induced widening of the leaving group-binding site enables ARH3 to specifically bind and cleave substrates with a structurally diverse leaving group (Figs. 1a and 4), supporting ARH3’s broad substrate specificity.

The flexible Glu\textsuperscript{41}-flap dynamically switches between closed and open conformations and plays important roles in substrate recognition (Fig. 6). In the unliganded ARH3, Glu\textsuperscript{41} of the Glu\textsuperscript{41}-flap serves as the key residue that constitutes the closed enzyme state by masking Mg\textsuperscript{B}. In the PAR substrate-bound state, Glu\textsuperscript{41} is released from Mg\textsuperscript{B} and instead interacts both with ADPR and the leaving group (Fig. 6), which presumably contributes to the precise alignment of the scissile O-linkage for subsequent cleavage and constitutes the open enzyme state. Therefore, we propose the Glu\textsuperscript{41}-flap as the gate that controls substrate entrance to the active site. Given the structural flexibility and solvent accessibility of the Glu\textsuperscript{41}-flap, it is also possible that the Glu\textsuperscript{41}-flap functions as a protein–protein interaction module. Proteins that specifically interact with the closed conformation of the Glu\textsuperscript{41}-flap of ARH3 might conformationally lock ARH3 in an inactive state and thereby regulate ARH3 function.

A notable ARH3 inhibition by ADP-HPD (Fig. S6a) (30), an analog of the oxocarbenium ion intermediate, raises the possibility that the ARH3 mechanism might involve an oxocarbenium intermediate in the distal ribose\textsuperscript{O} of ADPR in a similar way to PARG (15, 31), which is followed by water-mediated nucleophilic attack at the anomic C1\textsuperscript{O} position. The observed 18\textsuperscript{O} incorporation to C1 during hydrolysis of O-acetyl-ADPR (24) is also consistent with this model. In glycosidase superfamily members, a catalytic acidic residue is typically found near the scissile bond, e.g. Glu\textsuperscript{756} in rat PARG (Glu\textsuperscript{752} in human PARG) (31, 32), to activate a water molecule for nucleophilic attack on the anomic carbon (33). In the ARH3-ADPR-Mg\textsuperscript{2+} complex, Asp314 is located proximal to both 1\textsuperscript{-}OH (corresponding to the scissile 1\textsuperscript{-O}-linkage) and the axial water ligand (W1) of Mg\textsuperscript{B} (Fig. 3c). It is plausible that Asp314 might function as a general acid or base to protonate the leaving group and then activate W1 for backside attack of the anomic C1\textsuperscript{O} in a manner similar to Glu\textsuperscript{756} in rat PARG (Fig. S6b) (31). In support of this catalytic role of Asp314, the ARH3-D314N mutant shows a dramatic reduction in ARH3 activities (22, 25). Although more work is needed to determine the catalytic mechanism of ARH3, it is unlikely that ARH3 has a redox chemistry step for catalysis given its preference for Mg\textsuperscript{2+} over Mn\textsuperscript{2+} (Fig. 1b).

ARH3 is a unique multitasking enzyme that regulates cellular concentrations of both PAR, either free or attached to proteins, and mono(ADP-ribosyl)ated substrates. Elevated PARylation levels are often found in many human cancers, including...
ARH3 switches between closed and open conformations to specifically recognize and cleave substrates. ARH3 switches between a self-inhibited and an incision-competent conformation for specific substrate recognition and cleavage. In the absence of PAR substrates, the Glu<sup>41</sup>-flap caps Mg<sup>2+</sup>, constituting a closed, self-inhibited enzyme state. Substrate binding induces a conformational switch in the Glu<sup>41</sup>-flap, allowing substrate entrance to the active site. This active-site rearrangement in ARH3 specifically exposes the scissile 1'-O-linkage to the catalytic Asp<sup>114</sup> for cleaving, explaining ARH3 specificity for the 1'-O-linkage in structurally diverse substrates.

**Experimental procedures**

**Plasmids and protein purification**

Human ARH3<sup>FL</sup> was cloned into a modified pET21b vector with an N-terminal His<sub>6</sub> tag and a following cleavage site for PreScission protease (pET21b-His<sub>6</sub>-pps). The pET21b-His<sub>6</sub>-pps-ARH3<sup>FL</sup> plasmid was introduced into *Escherichia coli* Rosetta 2 (DE3) cells, and ARH3<sup>FL</sup> was induced by adding 1 mM isopropyl β-D-thiogalactoside overnight at 16 °C. ARH3<sup>FL</sup> was purified by affinity capture on a nickel-nitrilotriacetic acid (GE Healthcare) column. After elution with imidazole (250 mM), the protein was loaded onto a heparin column (GE Healthcare) and eluted with a NaCl gradient (0.1–1 M). Fractions with ARH3<sup>FL</sup> were pooled, and PreScission protease was added to cleave the N-terminal histidine tag. Finally, ARH3<sup>FL</sup> was loaded to a Sephacryl 200 (GE Healthcare) size-exclusion column using a buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, and 5% glycerol. The purified ARH3<sup>FL</sup> was concentrated to ∼30 mg/ml and then stored at −80 °C. A gene encoding the ARH3-D314E mutant was synthesized (GeneUniversal, Inc.) and cloned into pET-21b-pps vector. The ARH3-D314E mutant was purified using the same protocol as the WT protein. The human PARG catalytic domain (residues 448–976) was cloned in pET21b-His<sub>6</sub>-pps, expressed in *E. coli* Rosetta (DE3) cells, and purified by nickel-nitrilotriacetic acid–affinity chromatography, heparin chromatography, and Sephacryl 200 size-exclusion chromatography as described previously (32). For preparation of PARylated PARP1, the DNA-binding domain (residues 1–374) of human PARP1 and the PARP1C catalytic domain (residues 375–1014) were purified as described previously (6, 31).

**Crystallization and data collection**

The WT and the D314E mutant of ARH3 (10 mg/ml) were cocrystallized with 5 mM ADPR by hanging-drop vapor diffusion. ADPR binding is required for crystallization as the unliganded ARH3<sup>FL</sup> did not yield any crystals. The protein solution was mixed with an equal volume of well solution (22% PEG 4000, 0.1 M sodium acetate, pH 4.5, and 0.1 M MgSO<sub>4</sub>) and incubated at 22 °C. Crystals were briefly equilibrated in a harvest solution (26% PEG 4000, 0.1 M sodium acetate, pH 4.5, 0.1 M MgSO<sub>4</sub>, and 5 mM ADPR), transferred to a cryoprotectant solution (26% PEG 4000, 0.1 M sodium acetate, pH 4.5, 0.1 M MgSO<sub>4</sub>, 5 mM ADPR, and 15% glycerol), and then flash-cooled in liquid nitrogen for data collection.

X-ray diffraction data were collected at the NE-CAT 24ID-E beamline at the Advanced Photon Source. The ARH3<sup>WT</sup>–ADPR–Mg<sup>2+</sup> complex crystals (P1 symmetry; four ARH3–ADPR–Mg<sup>2+</sup> complexes per asymmetric unit) diffracted to

**Figure 6.** ARH3 switches between closed and open conformations to specifically recognize and cleave substrates. ARH3 switches between a self-inhibited and an incision-competent conformation for specific substrate recognition and cleavage. In the absence of PAR substrates, the Glu<sup>41</sup>-flap caps Mg<sup>2+</sup>, constituting a closed, self-inhibited enzyme state. Substrate binding induces a conformational switch in the Glu<sup>41</sup>-flap, allowing substrate entrance to the active site. This active-site rearrangement in ARH3 specifically exposes the scissile 1'-O-linkage to the catalytic Asp<sup>114</sup> for cleaving, explaining ARH3 specificity for the 1'-O-linkage in structurally diverse substrates.

**BRCA-deficient breast cancers** (34, 35) and triple-negative breast cancers (36, 37). ARH3<sup>−/−</sup> cells undergo enhanced PARylation-dependent cell death upon genotoxic stresses but remain healthy under unstressed conditions. We suggest that pharmacological intervention in ARH3-dependent pathways could be a safe and efficient therapeutic strategy for cancers with up-regulated PARylation by increasing the cytoplasmic PAR level and triggering parthanatos, either alone or in combination with current chemotherapeutic agents. Our structures reveal extensive adenine interactions with the adenine cap and a large conformational switch of the Glu<sup>41</sup>-flap that are crucial for specific engagement of structurally diverse substrates. These unique structural elements of ARH3 can be exploited for the development of specific ARH3 inhibitors, which have potential therapeutic applications, as well as the ability to advance our understanding of the role of ARH3 in regulation of PARylation. A concern is that ARH3 also catalyzes hydrolysis of O-acetyl-ADPR, which may be involved in other cellular pathways such as chromatin remodeling. This activity also relies on the ability of ARH3 to act at the C1' position of ADPR. ARH3's contribution to the O-acetyl-ADPR–dependent pathways, in contrast to other proteins in the ARH-macromdomain superfamily, is not known. Thus, some ARH3 inhibitors may affect multiple signaling pathways due to its multiple enzymatic activities and substrates.
Structure of human ARH3 bound to ADP-ribose and Mg\(^{2+}\)

1.7-Å resolution, and ARH3\(^{D314E}\)–ADPR–Mg\(^{2+}\) complex crystals (P1 symmetry; four ARH3–ADPR–Mg\(^{2+}\) complexes per asymmetric unit) diffracted to 1.6-Å resolution. X-ray data sets were collected with an Eiger 16M detector and processed using HKL2000 (38) and SCALEPACK (38, 39). Data collection statistics are shown in Table S1.

Structure determination

The full-length human ARH3\(^{WT}\)–ADPR complex structure was determined by molecular replacement using MolRep (40) in the CCP4 suite with the apo-ARH3\(^{3N16}\) structure (25) as a search model. The asymmetric unit contains four ARH3 molecules, and they all show a strong difference electron density for the bound ADPR at the active site, which was supported by polder map calculations. The restraints for ADPR were generated using Monomer Library Sketcher in the CCP4i suite (41). The model was manually rebuilt using Coot (42) and refined with PHENIX (43) to an R\(_{\text{factor}}\) of 18.4% and an R\(_{\text{free}}\) of 21.8%. The ARH3\(^{D314E}\)–ADPR complex structure was determined by molecular replacement using MolRep (40) with the ARH3\(^{WT}\)–ADPR–Mg\(^{2+}\) complex structure as a search model. The ARH3\(^{D314E}\)–ADPR–Mg\(^{2+}\) complex model was rebuilt using Coot (42) and refined with PHENIX (43) to an R\(_{\text{factor}}\) of 16.7% and an R\(_{\text{free}}\) of 19.9%. Crystallographic data statistics are shown in Table S1. The Ramachandran plot shows that >98% of the residues in both ARH3\(^{WT}\)–ADPR–Mg\(^{2+}\) and ARH3\(^{D314E}\)–ADPR–Mg\(^{2+}\) complexes are in the favored regions, and all the others are in the allowed regions. No outlier residue was observed.

ARH3 activity assay

ARH3 activity was measured against PARYlated PARP1 using a method similar to that for PARG activity measurement as described previously (31). The C-terminal catalytic domain of PARP1 (PARP1C; residues 375–1014) containing the auto-modification domain was PARYlated in the presence of the N-terminal DNA-binding domain of PARP1, a nicked DNA, and NAD\(^{+}\) as described previously (6, 31). Briefly, PARYlation of PARP1 was performed at 37 °C in a reaction buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 2 mM DTT. To PARYlate PARP1, PARP1C (2 μM), DNA-binding domain (residues 1–374) (2 μM), and a nicked DNA (2 μM) were preincubated for 10 min on ice. 200 μM NAD\(^{+}\) was then added to the reaction, and it was then incubated at 37 °C for 30 min. After desalting with a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 5% glycerol, purified human ARH3 proteins (WT and D314E) or human PARG was treated with PARYlated PARP1 substrates in the presence or absence of 5 mM EDTA or divalent metals (Mg\(^{2+}\), Mn\(^{2+}\), and Ca\(^{2+}\)) and incubated for 60 min at 37 °C. The level of modification of PARP1 was visualized by Coomassie Blue staining of SDS-polyacrylamide gels.

Computational modeling of the ARH3–di-ADPR–Mg\(^{2+}\) complex

The di-ADP-ribose model was generated by covalently linking 2’-OH of the adenosine ribose of n − 1 ADPR to the anomic C1’ of the distal ribose of n ADPR in the ARH3–ADPR–Mg\(^{2+}\) complex using YASARA (44). The n ADPR molecule, two Mg\(^{2+}\) ions, and the bridging μ-aqua ligand remained anchored to the experimentally identified site in the ARH3–ADPR–Mg\(^{2+}\) complex. Then n − 1 ADPR was docked to the putative n − 1 ADPR-binding site (leaving group–binding site) of the J-shaped substrate-binding channel of ARH3. The ARH3–di-ADPR–Mg\(^{2+}\) model was then subjected to energy minimization using the AMBER ff14SB protein force field in the CHIMERA package (45). All atoms in the protein and di-ADPR ligand were allowed to move during energy minimization. All structural figures were prepared using PyMOL.

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