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A family of killer toxins

Exploring the mechanism of ADP-ribosylating toxins

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The ADP-ribosylating toxins (ADPRTs) are a family of toxins that catalyse the hydrolysis of NAD and the transfer of the ADP-ribose moiety onto a target. This family includes many notorious killers, responsible for thousands of deaths annually including: cholera, enterotoxic *Escherichia coli*, whooping cough, diphtheria and a plethora of *Clostridial* binary toxins. Despite their notoriety as pathogens, the ADPRTs have been extensively used as cellular tools to study and elucidate the functions of the small GTPases that they target. There are four classes of ADPRTs and at least one structure representative of each of these classes has been determined. They all share a common fold and several motifs around the active site that collectively facilitate the binding and transfer of the ADP-ribose moiety of NAD to their protein targets. In this review, we present an overview of the physiology and cellular qualities of the bacterial ADPRTs and take an in-depth look at the structural motifs that differentiate the different classes of bacterial ADPRTs in relation to their function.

Pathogenic bacteria are known to possess an arsenal of toxins and effectors that assist them in targeting and killing their host cells. The ADP-ribosylating toxins (ADPRTs) are a large family of dangerous and potentially lethal toxins. Examples of these toxins can be found in a diverse range of bacterial pathogens and they are the principal causative agents in many serious diseases including cholera, whooping cough and diphtheria. ADPRTs, as the name would suggest, break NAD into its component parts (nicotinamide and ADP-ribose) before selectively linking the ADP-ribose moiety to their protein target (Fig. 1). In the majority of these toxins, the targets are key regulators of cellular function and interference in their activity, caused by ADP-ribosylation, leads to serious deregulation of key cellular processes and in most cases, eventual cell death.

This large family of toxins has been extensively studied with many structures of individual members determined. These include: diphtheria toxin (1TOX) [1], pseudomonas exotoxin A (1AER) [2], pertussis toxin (1PRT) [3], cholera toxin (1XTC) [4], *Escherichia coli* heat labile enterotoxin (1LTS) [5], Iota toxin (1GIQ) [6], vegetative insecticidal protein (1QS1) [7] and the C3-like toxins, C3bot (1G24) [8] and C3stau (1OJZ) [9]. These structures and extensive cellular and functional research performed over the last 20 years have provided an enormous insight into the function of these toxins and an understanding of their effects on host cells. These data are summarized in Table 1. The

Abbreviations
ADPRT, ADP-ribosylating toxin; ARF, ADP-ribosylation activation factor; ART, ADP-ribosyltransferase; ARTT, ADP-ribosyl turn-turn; CT, cholera toxin; DT, diphtheria toxin; eEF2, elongation factor 2; LT, *E. coli* heat labile enterotoxin; NMN, nicotinamide mononucleotide moiety; PARP, poly-ADP-ribose polymerase; PAETA, *Pseudomanas aeruginosa* exotoxin A; PT, pertussis toxin; STS motif, *Aromatic*-hydrophobic-serine-threonine-serine motif; VIP2, vegetative insecticidal protein.

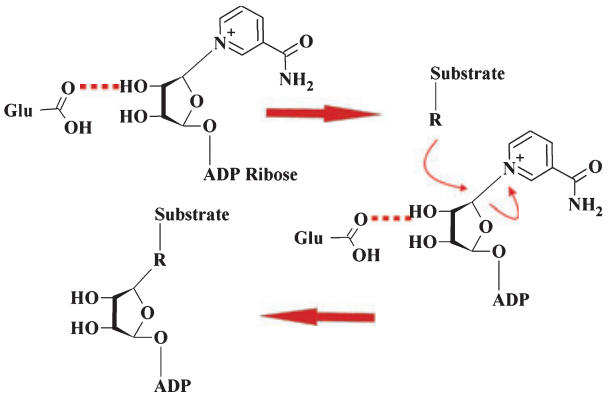

Fig. 1. A generalized mechanism of ADP-ribosylation. NAD is bound to the toxin and the catalytic glutamate forms a hydrogen bond with the 2′-OH of the ribose. This hydrogen bond stabilizes the active intermediate and leaves the *N*-glycosidic bond vulnerable to nucleophilic attack from the target. This results in ADP-ribose being covalently bonded to the target.

ADPRT family can be split into four groups on the basis of their domain organization and the nature of their target. The 3D structure of a representative member of each group is shown in Fig. 2. The most well known toxins: cholera, pertussis and the *E. coli* enterotoxin are members of the AB_5 family which target small regulatory G-proteins. The enzymatically active A subunit is situated on a scaffold made of a pentamer of B-subunits [4,10–14]. Diphtheria and Pseudomonas exotoxin A ribosylate a diphthamide residue on elongation factor 2. Both are large multidomain proteins with receptor binding, transmembrane targeting and protease-resistant catalytic domains [15–20]. The third group are the actin-targeting AB binary toxins that, unlike the more common AB_5 binary toxins, comprise of two domains, an active catalytic domain and a cell-binding domain. This group includes a wide range of clostridial toxins including C2 toxin from *Clostridium botulinum*, *Clostridium perfringens* Iota toxin, *Clostridium spiroforme* toxin, *Clostridium difficile* toxin and the vegetative insecticidal protein (VIP2) from *Bacillus cereus* [7,21–24]. The final group are the small single domain C3 exoenzymes that have an unknown role in bacterial pathogenesis, but are widely used as tools in cellular signalling work, and are characterized by C3bot from *C. botulinum*. This group also includes similar enzymes from *Clostridium limosum*, *B. cereus* and *Staphylococcus aureus* [25–30].

Several ADPRT structures determined to date have been elucidated in the presence of bound NAD molecule or nonhydrolysable NAD analogues and these have allowed a detailed understanding of NAD binding. These structures combined with biochemical results have also suggested a possible catalytic mechanism. The current understanding of the mechanism of catalysis is that NAD is bound by the ADPRT in a

Table 1. Summary of the ADPRTs that have had their 3D structures determined, giving their targets and physiological effects.

Toxin	Organism	PDB ID	Class	Target	Effects
Pertussis toxin	*Bordetella pertussis*	1PRT	AB_5	Cysteine on G_i, G_t and G_a	Uncoupling of effectors from the adenylate cyclase pathway
Cholera toxin	*Vibrio cholerae*	1XTC	AB_5	Arg on G_s	Trapping of G-protein in GTP bound states and uncontrolled up-regulation of adenylate cyclase
E. coli heat labile enterotoxin	*Escherichia coli*	1LTS	AB_5	Arg on G_s	Trapping of G-protein in GTP bound states and uncontrolled up-regulation of adenylate cyclase
Diphtheria toxin	*Corynebacterium diphtheriae*	1TOX	AB Three domain	Diphthamide on eEF2	Inhibition of protein synthesis
Pseudomonas exotoxin A	*Pseudomonas aeruginosa*	1AER	AB Three domain	Diphthamide on eEF2	Inhibition of protein synthesis
VIP2	*Bacillus cereus*	1QS1	AB binary toxin	Arg177 on Actin	Prevent actin polymerization
Iota toxin	*Clostridium perfringens*	1GIQ	AB binary toxin	Arg177 on Actin	Prevent actin polymerization
C3bot	*Clostridium botulinum*	1G24	Single polypeptide	Asn41 on Rho A-C	Trap Rho GTPase in GDP bound state and leads to disaggregation of actin cytoskeleton
C3stau	*Staphylococcus aureus*	1OJZ	Single polypeptide	Asn41 on Rho A-C, RhoE and Rnd3	Trap Rho GTPase in GDP bound state and leads to disaggregation of actin cytoskeleton
Ecto-ART2	Rat	1OG1	Single polypeptide	Arg residue on integrins	Cell regulation and a role in apoptosis

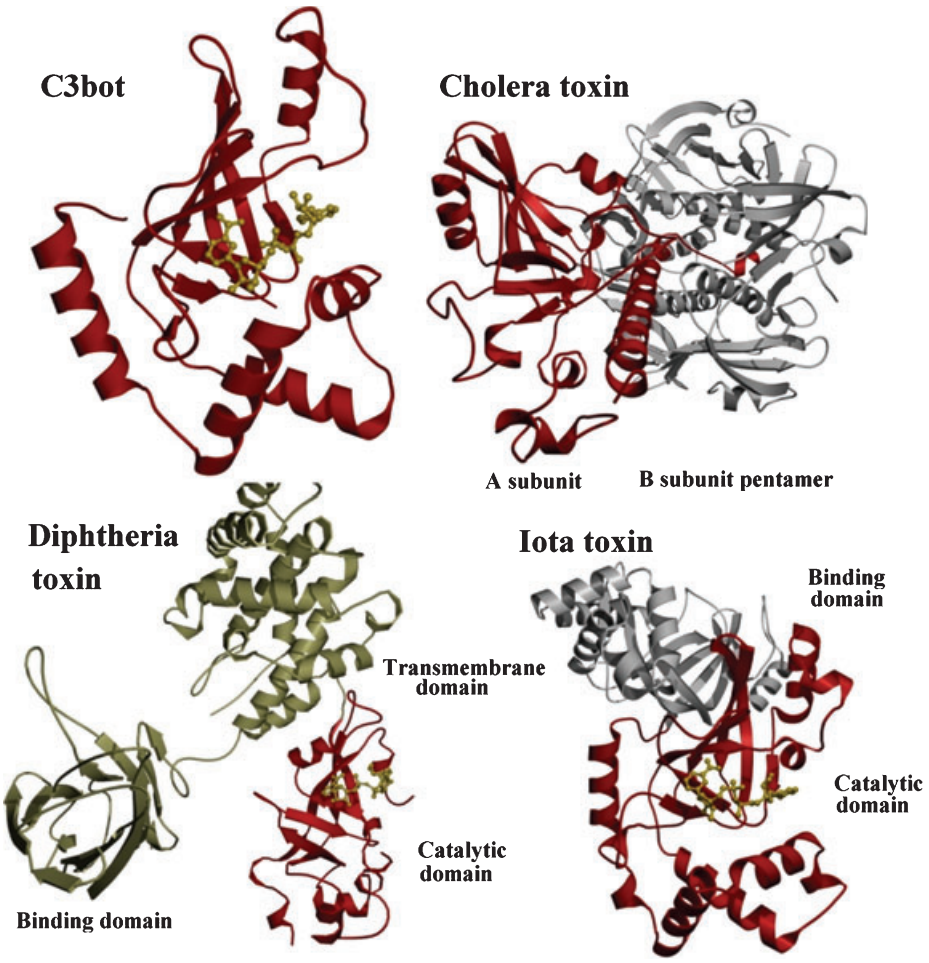

Fig. 2. Structures of the C3-like (top left) [8], DT (bottom left) [1], Iota-like (bottom right) [6] and CT (top right) [4] classes demonstrating the domain organization and architecture of the different classes of ADPRTs. In all frames, the catalytic unit bearing the ADPRT activity is highlighted in red. Figures were made using MOLSCRIPT [88].

manner that orients the glycosidic bond to render it amenable to hydrolysis. The 'catalytic glutamate residue', that all known ADPRTs possess, forms a hydrogen bond with the 2′-OH of the ribose ring and this can be seen for all three classes in Fig. 3A–C. This interaction stabilizes a positively charged oxocarbenium ion intermediate, which is then attacked by a nucleophile, either an activated water molecule in the case of auto-hydrolysis that many ADPRTs demonstrate, or the protein substrate. A simplified version of this mechanism is shown in Fig. 1. While the toxin-substrate recognition process has still not been fully understood, through biochemical and mutagenic analysis there are some elements of protein-recognition that are known. In the case of cholera, pertussis, the *E. coli* enterotoxin and the diphtheria toxin (DT) family of toxins, an 'active site loop' has been shown to be essential for substrate binding [31]. An example of such a detailed interaction has been provided by the recently determined crystal structure of the elongation factor 2 (eEF2) and *Pseudomanas aeruginosa* exotoxin A (PAETA) complex [32] in which the active site loop (L4 in PAETA) plays an important role. Likewise, the active site loop has been shown to be essential for activity in cholera toxin, *E. coli* enterotoxin and the distantly related ExoS and T toxins [1]. The recent structure of the complex between the activated form of cholera toxin (CT) and a human ADP-ribosylation activation factor 6 (ARF6) may suggest the mechanism of this active site loop involvement. In the activated ARF6-CT complex, the binding of ARF6 causes an allosteric change on the CT toxin that results in the active site loop forming an extended 'knob' near the ADP-ribosyl turn-turn (ARTT) loop altering the structure of the CT active site into a more suitable one for substrate binding and ADP-ribosylation. In the C3 and Iota-like toxins, the

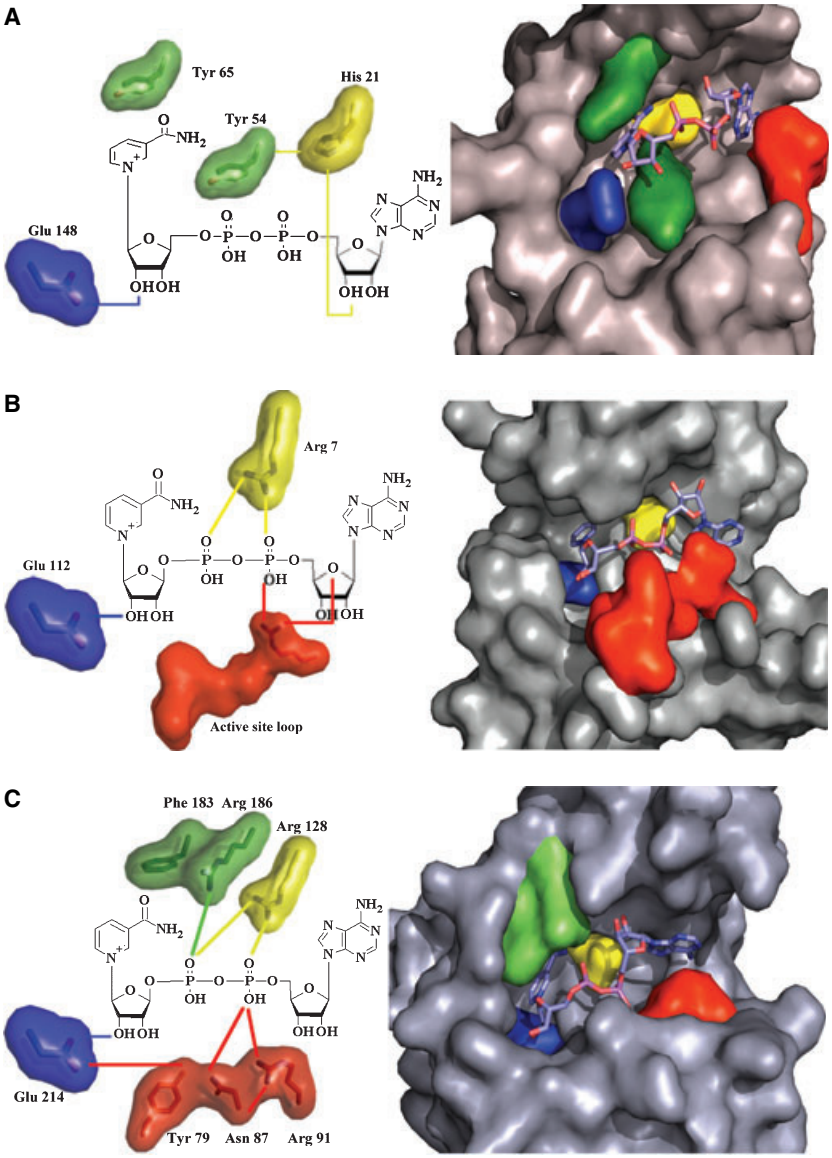

Fig. 3. (A) Schematic view of the active site cleft of the DT class of toxins highlighting the key catalytic residues and mode of NAD binding [1]. This illustrates the stacking of the nicotinamide ring between the conserved tyrosines, the binding of the conserved His to the O_2 of the adenine ribose and the carbonyl oxygen of Y54 and the binding of the conserved catalytic glutamate to the O_2 of the nicotinamide ribose. (B) Schematic view of the NAD binding cleft of the cholera-like class of toxins showing the intramolecular interactions around the active site and the key features [4]. The binding of R7 to the oxygens of the NAD when in the active state instead of the carbonyls of R54 and S61 in the inactive form can clearly be seen. An arginine from the active site loop, in its active form, is also involved in binding the phosphate-oxygens and the ribose ring of the adenine. (C) The important residues and bonds formed around the NAD binding site by the four motifs found in the α-3 type toxins [8]. The α-3 asparagine and arginine bind the phosphate oxygen, holding the NAD in a compact state. This is the role also undertaken by the conserved arginines of the PN loop and Arg/His motif. The catalytic glutamate and its stabilizing bond from the tyrosine of the α-3 motif are also shown.

details of substrate recognition are less clear, though all of them possess an aromatic residue on the ARTT loop that has been found to be critical for substrate binding [33]. The conserved Q/E residue that is found two residues upstream of the catalytic glutamate in the ADP-RTs may also play a role in substrate specificity. The Rho-binding toxins all possess a Q-x-E motif, whilst the actin binding toxins possess an E-x-E motif as shown in the sequence alignment in Fig. 4A. The change between Q and E has also been demonstrated to have substrate-altering properties in the eukaryotic Ecto-ART proteins [34]. However, as only one toxin–substrate complex has been determined so far, the exact mechanism and the process of protein–protein recognition still remain much of a mystery.

Cellular properties of ADPRTs

The bacterial ADPRTs are all thought to play important roles in bacterial pathogenesis acting as key virulence factors in many diseases. The disruption caused by the ADPRTs varies considerably between the four classes, but all rely on the ADP-ribosylation of key regulatory proteins in the host cells to disrupt cell signalling and interfere with downstream regulatory and structural processes.

The AB_5 proteins [pertussis toxin (PT), *E. coli* heat labile enterotoxin (LT) and CT] all ribosylate a small subsection of the G-protein family. In all three toxins, the A1 catalytic domain sits on top of a doughnut shaped pentamer of binding domains that

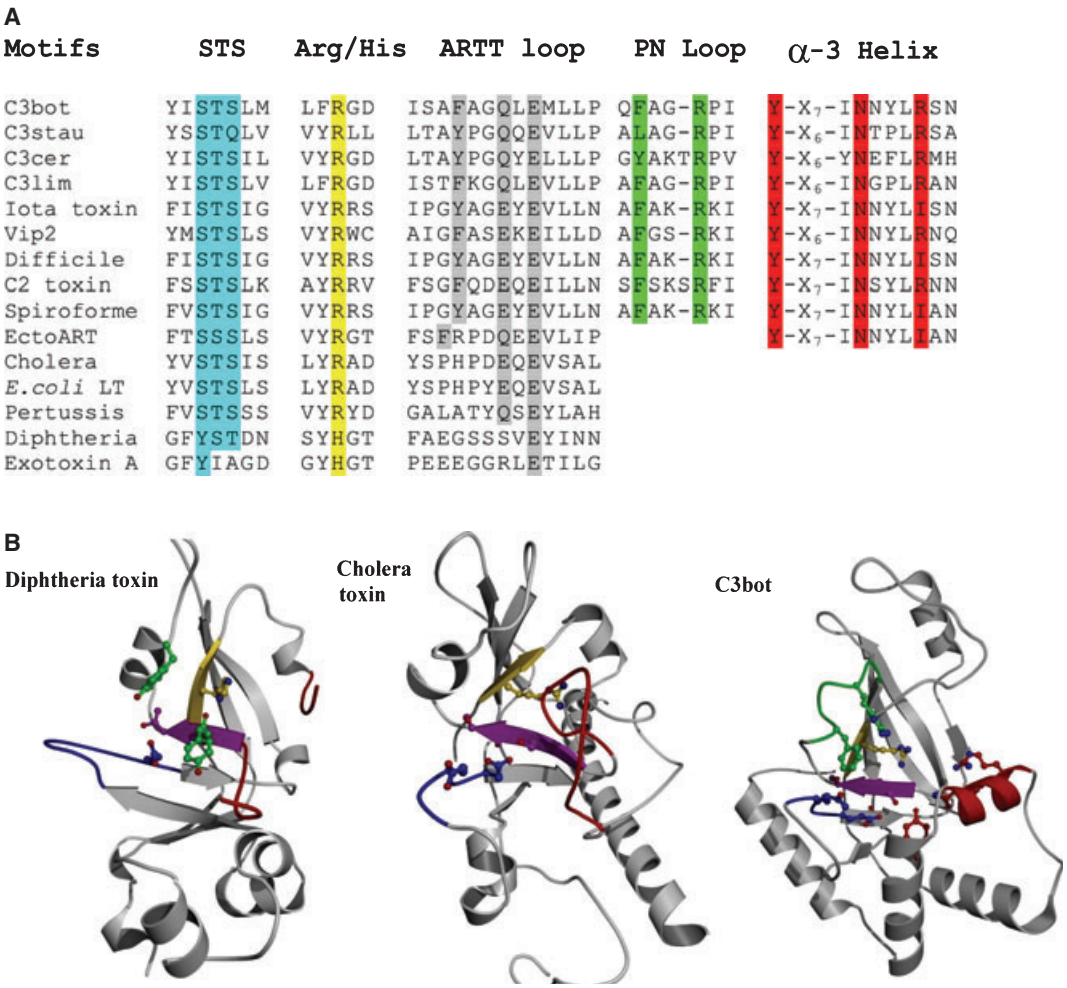

Fig. 4. (A) Sequence alignment of the three classes of ADPRT highlighting the conserved residues that make up each of the motifs. The conserved residues in each motif are shaded in the same colours used in Fig. 4B. (B) Ribbon diagrams of a diphtheria-like [1], cholera-like [4] and α-3 [8] toxins highlighting the important motifs in each molecule. The glutamate containing ARTT loop is highlighted in blue, with the STS and Arg/His motif in purple and yellow, respectively. The active site loops are shown in red, as is the α-3 helix. Shown in green is the PN loop for the α-3 toxins and the Ty-X_{10}-Tyr motif for the DT toxins. In all cases, figures were generated using MOLSCRIPT [88].

comprise the cell binding and translocation apparatus [3,4,35–37]. In the bacterial cell, this hetero-hexamer is assembled in the bacterium before being transported across the membrane via the type II secretion apparatus [38]. Once secreted into the lumen of the gut, the B-pentamer recognizes the GM−1 ganglioside on the host cell surfaces inducing endocytosis and translocation into the cytosol. Trafficking and processing of the full holotoxin in the host cell is a tremendously complex process and the description is outside the scope of this review. For the toxin to become active, however, the catalytic domain must undergo proteolytic cleavage of the disulphide linked A1–A2 domain before becoming fully active [12,39]. This also results in the A1 domain being released from the A2–B_5 complex. Even then these toxins are not fully functional and require activation by host cell proteins to become fully active. In the case of cholera toxin, these ADP-ribosylation activation factors (ARFs) come from the host and are small GTPases that bind the CT in their GTP-bound state.

Both CT and LT target the $G_{S\alpha}$, the stimulatory G-protein of the adenylate cyclase system. ADP-ribosylation of this causes the G-protein to be maintained in its activated GTP bound state [40] and leads to a massive up-regulation of adenylate cyclase and subsequent increase in the amount of cystolic cyclic AMP [12,41]. This eventually leads to a major loss of fluids and ions from the affected intestinal cells and gives rise to the severe diarrhoea and fluid loss

associated with both cholera and enterotoxigenic *E. coli* pathogenesis [42,43].

PT is one of the primary virulence agents produced by *Bordetella pertussis,* the major causative agent of whooping cough. Pertussis toxin ADP-ribosylates an exposed cysteine residue on several small heteromeric G-proteins; the most prominent examples are $G_{i\alpha}$, $G_{o\alpha}$ and $G_{t\alpha}$ [44,45]. This results in uncoupling of the G-proteins from their effectors and an unregulated increase in adenylate cyclase activity and an increase in cyclic AMP [46]. As many cells possess PT receptors, the physiological effects of PT pathogenesis vary greatly from one cell type to another.

DT and PAETA are both examples of eEF2 ribosylating toxins [20]. Upon cell entry they both specifically ribosylate an exposed histidine that has been modified by the addition of a diphthamide side-group [47]. The ADP-ribosylation interrupts the function of eEF2 in the host cell interfering with protein synthesis which results in profound physiological changes and ultimately cell death [19,48]. The events leading up to this point are well understood, and appear to rely on the action of the receptor binding and transmembrane targeting domains. PAETA binds to the α_2-macroglobulin receptor on the cell surface and induces receptor-mediated endocytosis, becoming internalized into endosomes where the low pH creates a conformational change in the toxin leaving it open to furin protease cleavage that removes the binding domain. The catalytic domain then undergoes retrograde transport to the endoplasmic reticulum, translocates into the cytoplasm and can enzymatically ribosylate eEF2. DT by contrast binds to the epidermal growth factor-like growth factor precursor (HB-EGF) and is cleaved on the cell surface before uptake through receptor mediated endocytosis. Once in the early endosome, the DT catalytic fragment is not processed and penetrates the membrane of the endosome to pass directly into the host cell cytoplasm where it can ribosylate eEF2.

Iota toxin, from *Clostridium perfringens* [22], and VIP2 from *Bacillus cereus* [7] are both actin ADPRTs, each ribosylating actin at an exposed arginine, Arg177 [49]. The ADP-ribosylation prevents actin polymerization by capping the exposed ends of the actin filaments which leads to cell rounding and eventual cell death as the actin cytoskeleton breaks down [50]. This class of actin modifying binary toxins also includes *C. botulinum* C2 toxin [21], *C. spiroforme* toxin [23] and *C. difficile* toxin components cdtA and cdtB [51]. The domain structure of Iota and VIP2 is also of interest, as the two domains resemble one another closely. The second domain is responsible for cell binding and lacks catalytic activity, suggesting that the binary toxins may have arisen from gene duplication of an original ADP-RT ancestor [7]. The toxins do not bind cells as complete A–B units. Instead proteolytically activated B monomers bind to cell surface receptors as homo-heptamers. These homo-heptamers then bind the A domains and are taken into cells via endocytosis. Once inside acidic endosomes, the low pH activates the translocation function of the B domain heptamers and they translocate the catalytic A domains across the endosomal membrane into the cytoplasm where they can act to ribosylate actin and bring about cell death [52].

The C3 exoenzymes are characterized by C3bot first identified from *C. botulinum* [26], but also include representatives from *Clostridium limosum* (C3lim) [27], *B. cereus* (C3cer) [53] and *S. aureus* (C3EDIN, C3Stau2) [29,30]. This family of ADPRTs selectively ribosylates the small GTPases, Rho A, B and C [54] at an exposed Arg41 [55]. This reaction is highly specific to only those substrates, except in the case of C3stau2, which has a slightly broader specificity that includes RhoE and Rnd3 [33,56]. The ADP-ribosylation prevents Rho moving into its active GTP-bound state and leads to a loss of control in the downstream pathways controlled by the Rho GTPases and resulting in loss of control of the cell cytoskeleton and eventual cell death [57]. Although these effects are in seen *in vitro*, the role of C3bot and its related ADPRTs in pathogenesis is not yet known as they lack any cell translocation or binding domains. C3stau, however, has been found in some clinical isolates and both C3bot and C3stau2 have been shown to prevent wound healing *in vivo* [58,59], suggesting that they may have some role in pathogenesis.

In addition to the selection of bacterial ADPRTs there are ADP-ribosyltransferases (ART) present in eukaryotic organisms. Eukaryotic ADP-ribosylation can be of two forms: (a) poly-ADP-ribosylation that is mediated by poly-ADP-ribose polymerases (PARPs) and catalyses the transfer of multiple ADP-ribose moieties onto a substrate; and (b) mono-ADP ribosylation that catalyses the transfer of a single ADP-ribose moiety onto a target and is mediated by Ecto-ADP ribosyltransferases (Ecto-ARTs) [60]. The PARP superfamily plays a role in the repair of DNA strand breaks and modulation of chromatin [60]. The structure of the catalytic domains of chicken PARP-1 and mouse PARP-2, however, did demonstrate structural homology to the active site of diphtheria toxin [61,62]. The five Ecto-ARTs found in mammalian systems, named Ecto-ART1–5, are located in the extracellular space of mammalian tissues and play a role in cell adhesion and the immune system. They are closer in structure to the C3-like ADPRTs than to the PARP family [63–66].

Structural analysis of the NAD binding site between ADPRTs

It has previously been demonstrated that all the ADP-RTs, both bacterial and the eukaryotic Ecto-ARTs, share a common active site and NAD binding motif [31,67]. With the determination of several ADPRT structures an examination of the active sites and interactions that are necessary for NAD binding and ribosyl transfer is possible. The structural analysis of several ADPRTs has led to them being classified into two groups that share a similar active site architecture but lack sequence homology. The 'DT' group is based on the active site and NAD binding features of Diphtheria toxin [1] and also includes *Pseudomonas* exotoxin A [16] and the mammalian PARPs [68]. The 'CT' group is based upon the NAD binding observed in CT [4] and includes: the LT [37]; PT [3]; C3bot [8]; VIP2 [7]; Iota toxin [6] and the Ecto-ART [34] family from eukaryotes [67]. With the determination of more ADP-RT structures it is now clear that the CT group should be divided further into those toxins that possess an active site loop involved in substrate binding [1] and those that instead have an α-helix forming part of the NAD binding cleft [8].

There are five key structural features that have been identified in the ADPRTs [67]. These are as follows: (a) the *Q/E-X-E* motif centred on the catalytic glutamate and the glutamate/glutamine responsible for the ribosyltransferase activity; (b) the *Arom-H/R* motif that contains either a histidine or an arginine that contributes to the NAD binding and maintains the structure of the active site cleft; (c) the *Aromatic*-hydrophobic-serine-threonine-serine motif (STS motif) on a β-strand that stabilizes the NAD binding; (d) the $Y\text{-}X_{10}\text{-}Y$ motif in DT and PAETA that fulfils the role of the STS motif in the bacterial ADPRT; and (e) the PN loop that contains *A/G-x-R-x-I* motif and is found in the Iota-like binary toxins and C3bot type ADPRTs. The PN loop is a flexible loop above the NAD binding site that creates a more compact binding site. It also brings into play an essential arginine residue which positions the NAD in a conformation more suitable for the cleavage of the nicotinamide N-glycosidic bond in these toxins [69]. The conserved sequences and physical position of these features are shown in Fig. 4A,B.

The ADPRT core fold

All the ADPRTs, both in the DT and the CT family, possess a near identical mixed α/β core structure of ~100 residues even though there is little sequence homology among many of them. This core structure has the approximate dimensions of $35 \times 40 \times 55$ Å and possesses the NAD binding site which supports both NAD glycohydrolytic and ribosyltransferase activities. The core is constructed from two perpendicular β-sheets with a variable number of α-helices attached to it both above and below the frame of β-sheets. The NAD binding site is positioned in a cleft made between the β-framework and either an α-helix in the case of C3bot, C3stau, VIP2, Iota and mammalian Ecto-ART or a variable length active site loop in pertussis, cholera, LT, diphtheria and exotoxin A. The latter is thought to be involved in EF2 or G-protein recognition [1].

The conserved motifs that characterize the ADPRT family

The ARTT motif

The ARTT loop contains the key catalytic glutamate responsible for the catalysis and transfer of the ribose moiety and the *Q/E-X-E* motif that is found in all members of the CT group [8,31,67]. The ARTT loop is of variable length comprised either of two sharp turns (turn 1 and turn 2) connecting either two β-sheets, as in the ARTT loop of Iota toxin [6] and C3bot [8] that connects β5 and β6, or a longer loop connecting an α-helix to a β-sheet as in cholera toxin, pertussis toxin and LT. The ARTT loop in the different types of ADPRT can be seen in Fig. 4(B). In all the ADPRTs that are members of the CT group, there is also a Glu or Gln residue two residues from the catalytic glutamate residue [31]. This second residue is vital for the ribosyltransferase activity of the ADPRT, but not necessarily the NAD glycohydrolysis activity [33,70]. In Ecto-ART, the equivalent residue, Gln187, has been implicated in changing the substrate from cell surface to cytoplasmic substrates [34]. This substrate selectivity has also been observed through mutational analysis of C3lim, where mutation from Gln to Glu altered the ADP-ribosylation target from asparagine to arginine [71]. This Gln/Glu residue may play a role substrate selection. As can be seen from the sequence alignment in Fig. 4A, the actin and G-protein modifying proteins that ribosylate an exposed arginine possess a glutamate residue. In the Rho GTPase ribosylating proteins that ribosylate an exposed asparagine, it is a conserved glutamine residue.

Another important residue on the ARTT loop is the aromatic group situated on the centre of the loop between turn 1 and turn 2 that is found in C3bot, C3stau, Iota, VIP2 and Ecto-ART. In C3bot, this has been shown to be essential for Rho substrate binding

[33] and, in the proposed model of C3bot-RhoA recognition [8], it is a vital determinant of substrate recognition and binding. This mode of action could apply to the other closely related ADPRTs. This aromatic group, proposed to be important in substrate binding, is only present in the actin and Rho GTPase-modifying toxins and Ecto-ART family. The C3, Iota-like and Ecto-ART enzymes have also substituted the active site loop with an α-helix at the NAD binding cleft. In CT and DT, the active site loop is involved with substrate selectivity and binding. This loop has been seen to become disordered and undergo conformational change upon binding of NAD in both DT and PAETA [1,2].

The STS motif

The STS motif forms part of the β-sheet that comprises part of the NAD binding cavity and follows the pattern: *Aromatic-Hydrophobic-S-T-S* [67]. The STS motif acts as an anchor to hold the NAD binding site together. In C3bot the contribution of the STS motif (Ser174, Thr175, Ser176) is well understood [8]. Ser174, the first 'S' of the motif forms hydrogen bond with the catalytic glutamate and a tyrosine residue (Tyr79) beneath the cleft to hold the glutamate in the correct position to catalyse the cleavage of NAD. This is also seen in the Ecto-ART2 structure with the STS (Ser147) forming a hydrogen bond with the catalytic glutamate (Glu189) [63]. Similar interactions are also observed in C3stau2 [9] and VIP2 [7]. Mutation of this serine residue in the *C. botulium* C2 toxin (structure not yet determined) [72] eliminates the transferase activity. However, in diphtheria and Iota toxins, mutation of this serine residue reduces activity but does not entirely abolish the glycohydrolytic activity [1,70], suggesting that while the serine residue of this motif plays an important role in stabilizing the catalytic glutamate it is not essential in all ADPRTs. The threonine residue forms additional hydrogen bonds with perpendicular β-strands to stiffen the active site. Ser176 in C3bot, the second 'S' of the motif, forms hydrogen bonds with the loop immediately following the STS β-sheet and also with the glutamine residue of the *Gln/Glu-x-Glu* motif keeping the ARTT loop and the glutamine in the correct orientation for the transferase reaction [8]. In C3stau2, the second serine is replaced with a glutamine residue that binds to the nicotinamide of NAD directly. This is different to other ADPRTs that possess serine where the 'S' has a role in forming intramolecular bonds with the catalytic glutamatic acid. Because of this, the STS motif is thought to have a less important role in C3stau than in other related C3-like enzymes [9].

In the DT group, the STS motif is either partially lost (diphtheria) or entirely lost (exotoxin A). In diphtheria toxin, the STS motif is replaced by an YTS motif, but both the T and S residues are in similar positions as they are in the other CT toxins and are likely to play a similar role. The tyrosine residue (Tyr54) is crucial to the diphtheria activity and is one of the two conserved tyrosines essential for NAD binding through aromatic ring π-orbital stacking [73–75]. This is also the case in exotoxin A, with Tyr470, though it lacks the serine or threonine residue of the YST motif. This ring stacking stabilizes the bound NAD and plays a similar role to that of the STS motif in other toxins; that of stabilizing and maintaining the structure of the active site [1,2].

The key Arg/His residue

The conserved Arg/His residue [67] is comprised of an aromatic residue followed by the Arg/His and has been found in all the ADPRTs to date. In the DT family, the motif is Tyr-His while the members of the CT family also include a Val/Leu before the aromatic residue and all have arginine not histidine. In the ADPRTs, the purpose of the Arg/His motif is NAD binding and maintaining the structure of the active site rather than actual involvement in either glycohydrolase or the transferase reaction. Though not directly involved in the catalysis, the presence of the Arg/His motif has been shown to be vital from mutagenesis studies on C3stau2 [33], LT [76], PT [77,78], CT [79] and Iota toxin [6] and it has been shown that loss of this arginine either abolishes transferase activity either severely (C3stau2) or completely (Iota, LT, CT, and PT) reduces the hydrolase activity as well. The exact role that this Arg/His residue plays in NAD binding varies between the toxins depending on whether they contain an active site loop (e.g. DT, PAETA, CT, LT and PT) or an α-3 helix (e.g. C3-like and Iota like binary toxins). In the 'active site loop group' the residue, His21/440 in diphtheria/PAETA, Arg7 in LT and CT and Arg9 in PT, does not play an important part of binding NAD but instead supports key parts of the active site to position them in the correct orientation to hydrolyse the NAD. In DT, the histidine forms a hydrogen bond [1] with one of the hydroxyl groups on the adenenine ribose ring and, more importantly, forms a bond with the backbone carbonyl of one of the tyrosine pair in DT (Tyr54). Through this bond, the tyrosine is orientated into the correct orientation to bind the NAD, and can be seen in Fig. 3A. Until the recent structure of the NAD-bound cholera toxin was determined [80], a similar active site stabilization

was thought to occur in LT and the related CT and PT toxins [5]. In the absence of NAD, the catalytic Arg7 forms hydrogen bonds with Ser61 of the STS motif and the main chain carbonyl of Arg54, an arginine that forms electrostatic bonds with both Glu110 and Glu112. This network of bonds plays an important role in stabilizing the active site. However, when the cholera toxin is activated by an ARF protein, the active site loop undergoes a large conformational shift. This results in Arg54 being unavailable to interact with Arg7 and has a slight effect on the position Arg7, enabling it to bind an oxygen from each of the NAD phosphates rather than stabilizing Ser61 as shown in Fig. 3B. Thus, Arg54 acts in a manner similar to that seen in the α-3 toxins by binding directly to the NAD phosphates and positioning the NAD in a suitable conformation for hydrolysis. In DT and LT, the conserved His/Arg occupy identical spatial positions and interact with the backbone carbonyls of analogous residues, Tyr54 in DT and Ser61 of LT, that are the first residues of the YST/STS motifs. They also support a network of interactions that maintain the structure of the active site. Upon activation, however, the role of the catalytic arginine in CT reverts to the manner seen in the α-3 toxins by binding directly to the NAD. In the α-3 toxins, the arginine forms hydrogen bonds with the phosphates of the NAD positioning them in a more compact manner than is found in the 'active site loop' ADPRTs. These hydrogen bonds serve two purposes: Firstly, they improve the binding of NAD to the toxin and, second, they hold the phosphates in a position where they can interact with the nicotinamide amide group NN7 of the nicotinamide mononucleotide moiety (NMN) [6]. This can then assume a ring-like conformation that is prevented from moving due to stacking interactions with the aromatic residue on the PN loop (Phe349 in Iota toxin, Phe183 in C3bot) and withdraws electrons from the nicotinamide ring amide, increasing the susceptibility of the *N*-glycosidic bond to cleavage [34]. Thus the role of the Arg/His in the α3-helix toxins is very different to their structural role in the DT toxins and the NAD-free CT toxins where the Arg/His holds the active site in the correct manner to facilitate NAD binding.

The Tyr-X_{10}-Tyr motif

The toxins of the DT group (diphtheria and exotoxin A) are different from those in CT group in several respects. The first difference is the absence of the Gln/Glu-x-Glu motif (both diphtheria and exotoxin A possess only the catalytic glutamate) and the second is the lack of the STS motif that the toxins of the CT group possess. Instead the DT group possess a pair of tyrosines that stack above and below the plane of the NAD moiety and contribute to binding via π-orbital interactions, as shown in Fig. 3A. The orbital stacking is of vital importance here and has been shown by mutagenesis studies of PAETA with Tyr470 Phe/Tyr481 Phe mutants still possessing enzymatic activity [81]. Aromatic ring stacking may explain the slightly different conformation that NAD possesses in the PAETA and diphtheria toxin structures compared with the structures of ADPRTs from the CT group [1,82]. The aromatic stacking also places the NAD molecule in a position suitable to interact with the catalytic glutamate with the anomeric carbon of ribose exposed to solvent available for nucleophilic attack. Many of the CT group from the binary and C3-like families that possess the PN loop have an aromatic residue that stacks against the nicotinamide ring in a similar manner to the first tyrosine of the Tyr-X_{10}-Tyr pair.

The PN loop

The PN loop was first identified in the C3bot-NAD structure [69] and forms an essential part of the NAD binding site apparatus. The PN loop is a flexible loop that occurs 10 residues after the STS motif, connecting strands β3 and β4, and it undergoes a large movement upon NAD binding, becoming more ordered in the process. In C3bot, the PN loop has two residues that contributes to the binding of NAD; Arg186 which forms a hydrogen bond with one of the phosphate groups of NAD and Phe183 which stacks against the nicotinamide ring of NAD, as can be seen in Fig. 3C. Mutational analysis of Arg186 has revealed that it is essential to NAD binding [69]. In the case of Phe183, the stacking is similar to that of Tyr65 from DT, Phe160 from Ecto-ART and Tyr481 from exotoxin A. In C3stau, the PN loop is present and retains the critical Arg residue (Arg150), but the aromatic residue is replaced by a leucine. In both Iota and VIP2, the PN loop is intact with an Arg residue (Arg352 and Arg400, respectively) that forms bonds with an NAD phosphate molecule and an aromatic residue (Phe349 and Phe397, respectively) that stacks directly above the nicotinamide ring. In a similar manner to C3bot, the important nature of the PN loop has been confirmed in Iota toxin with mutants of Arg352 and Phe349 showing no activity or strongly diminished activity [6]. The PN loop is found in the α-3 toxins, both the C3-like and Iota-like, and there are no analogous arginines or aromatic residues present among the members of the CT group that possess active site loops. The DT group possesses a conserved tyrosine in a similar

position to the aromatic residue of the PN loop's *Arom*-X_2-*R* motif, but lacks the conserved arginine that is involved in NAD binding.

The α-3 motif

Within the ADPRT family, the actin-binding enzymes and C3-like exoenzymes all lack a 15-residue active site loop that is found in the cholera- and diphtheria-like toxins [1,37]. In CT and LT, this loop is implicated in G-protein binding and occurs around residues 45–58 [80]. In DT and PAETA, this active site loop is called L4 and comprises residues 39–48 and 483–490, respectively. In its place, the Iota and C3-toxins [6–9] have an α-helix that packs tightly against the NAD cleft forming a more compact binding site. Amongst these ADPRTs there are three important residues that appear on this α-helix and are conserved amongst nearly all of the C3-like and Iota-like ADPRTs. These are: (a) a tyrosine residue that interacts with the 'S' of the STS motif and the catalytic glutamate through hydrogen bonds, (b) an asparagine residue, and (c) an arginine residue that form part of the adenine ring-binding pocket. These three residues form another motif specific to the C3bot-like ADPRTs that possesses the α-3 helix instead of the active site loop, including the Ecto-ART-2 [34]. From sequence alignment, the related C3cer and C3lim also seem to have the signature sequence *Y*-$X_{6/7}$-*N*-X_2-*L*-*R*, except in the case of Iota and *C. difficile* toxin, where the arginine residue is replaced with an isoleucine.

Tyr79 in C3bot [8] and Tyr78 in Ecto-ART [63] can be seen to form charge interactions with the serine of the STS motif (Ser174 and Ser146, respectively) and the catalytic glutamate (Glu214 and Glu189, respectively). Mutational analysis on the equivalent tyrosine (Tyr246) in Iota toxin [6] showed that loss of the tyrosine resulted in reduced glycohydrolytic and transferase activities. This Y-S-E network of interactions may act as a stabilizing influence on the catalytic glutamate residue to ensure that it is positioned correctly to bind the NAD molecule and in a suitable charge state to stabilize the positively charged oxocarbenium transition state intermediate. The arginine and aspargine residues are involved in binding to the adenine end of NAD with binding observed in C3bot (Arg91-hydrophobic packing with the adenine ring and Asn87 binding to one of the phosphates), C3stau (Arg48-binding to the adenine ring), VIP2 (Arg315) and Ecto-ART2 (Asn87 and Arg91). In Iota toxin, although the arginine at position 259 is replaced with an Ile, a downstream asparagine residue (Asn255) has been shown to bind to the phosphates at the adenine end of the molecule. This bond contributed by the α-3 helix can be observed in Fig. 3C. Mutation of this asparagine to alanine caused a significant drop in enzymatic activity [6]. This binding to the adenine moiety may help NAD binding and assist in holding the ADP-ribose$^+$ after cleavage of the N-glycosidic bond until the transferase reaction can take place. The interactions between the Asn and the phosphates may contribute to positioning the phosphates closer to the NMN ring in a similar manner to the arginines of the Arg/His motif and PN loop.

Catalytic model and mechanism

The conserved nature of the NAD binding site and key catalytic residues would suggest a common catalytic mechanism shared between all members of the ADPRT family. The nucleophilic attack occurs at the anomeric carbon of the nicotinamide ribose and results in the cleavage of N-glycosidic bond separating the ADP-ribose moiety from the nicotinamide ring. The manner in which this occurs is still not precisely understood. Mechanisms have been put forward for S_N1- and S_N2-type reactions, though recent biochemical data would indicate that the reaction is of an S_N1 type.

The S_N2 reaction was first suggested for DT [1] and has also been put forward for VIP2 [7], C3bot [8], pertussis toxin [83] and Ecto-ART2 [34]. In the S_N2 reaction, the attacking nucleophile may be the substrate arginine, diphthamide or asparagine, depending on the toxin involved, and may even be water in auto-hydrolysis. This nucleophile is then deprotonated by either the conserved glutamate in DT [1,84], or the downstream *Gln/Glu*-X-Glu in the suggested mechanisms for Iota, C3bot and VIP2 or in pertussis toxin by a catalytic His35 [83]. This activated nucleophile then attacks the anomeric carbon of the ribose ring which, due to the conformation of the NAD, has been exposed to the solvent forming a pentacoordinate oxocarbenium transition state intermediate. In the CT group, this intermediate is partially stabilized by the catalytic glutamate forming a hydrogen bond with the O_2 atom on the nicotinamide ribose. This makes the ring more electronegative, which stabilizes the positively charged oxocarbenium ion before the *N*-glycosidic bond is cleaved, completing the transfer of the ADP-ribose$^+$ moiety on to the substrate. In the 'α3-helix' toxins, the nucleophilic attack may also be aided by the interactions between the nicotinamide amide group and phosphate oxygens that withdraw electrons from the nicotinamide ring, making the *N*-glycosidic bond even more attractive to the attacking nucleophile.

An S_N1 reaction has also been proposed for catalysis of ADPRT [85,86], which has an isolated positively charged oxocarbenium intermediate stabilized in a pentacoordinate state with direct stabilizing electrostatic interactions from the catalytic glutamate and serine hydroxyl group. This oxocarbenium intermediate comes about after the breaking of the glycohydrolytic bond. In this case, the serine residue is conserved and found in the STS motif. In the S_N1 reaction mechanism, the ADP-ribose$^+$ stays bound to the protein until attack from the nucleophilic substrate. Of the two mechanisms, the S_N1 reaction seems to be favoured by the available biochemical data, especially the uncoupling of glycohydrolytic and transferase reactions. This was achieved through mutagenesis of the Glu/Gln residue (Glu338 in Iota or Gln212 in C3bot). The DT group only possesses the catalytic glutamate that, in the S_N1 reaction, is required to stabilize the oxocarbenium intermediate. However, members of this group possess the active site loop that is thought to be involved in substrate binding and could position and activate the diphthamide group for nucleophilic attack on the stabilized oxocarbenium intermediate. Recently, the idea of a compulsory order S_N1 reaction with NAD binding being required for substrate binding has been challenged by new findings demonstrating that the interaction between PAETA and EF2 follows a random-order reaction mechanism and that either NAD or EF2 can bind and the reaction takes place via a random third order S_N1 reaction [86,87]. This has also been observed through kinetic isotope exchange studies [85], which support a random-order S_N1 type reaction. A more recent development supporting the available biochemical data is the determination of the structure of the eEF2–TAD–PAETA complex that captures the ADPRT reaction at an intermediate stage [32]. Though this complex supports the reaction of eEF2 and PAETA via an S_N1 mechanism, the exact nature of the reaction is still unknown. In the complex structure, the distance between the diphthamide N3 and the anomeric carbon was much larger than 2.8 Å, as suggested by the kinetic isotope exchange work, inferring that a crucial step in the mechanism has still not been captured.

Conclusions

From this brief overview of the conserved features and motifs of the currently characterized ADPRTs, it is possible to observe important common features and some key motifs that are conserved amongst the individual families of ADPRTs; these can be seen in Fig. 4A,B.

The conserved ADPRT core structure with a network of perpendicular β-strands is present in all of the ADPRTs that have had their structure determined to date. This is also true of the catalytic glutamate that is found in both the DT and CT families. This residue occurs in an equivalent position in all the structures determined to date, where it is required to stabilize the positively charged oxocarbenium ion transition state.

The division between the CT and DT groups is not as clear as once thought. Although members of the DT group lack the *Q/E-x-E* motif, in the case of PAETA the STS motif, they possess a unique Y-X_{10}-Y motif and, importantly, contain an active site loop that is responsible for substrate recognition and binding. This 'active site loop' makes them similar in both structure and function to the members of the CT group that have active site loops. The CT group should be split into two groups: (a) the 'active site loop' toxins, including CT, PT, LT and (b) the 'α-3' toxins that possess an α-helix beside the active site rather than a loop. These include the Iota-like binary toxins, the C3-like toxins and the eukaryotic Ecto-ART family. The α3-toxins differentiate themselves from the 'active site loop' toxins in the PN loop and the α-3 helix, as well as the behaviour of the Arg/His motif.

The recent kinetic and biochemical studies of the eEF2–PAETA complex [32,85–87] have confirmed that, for the DT group, the reaction is a third order S_N1 reaction. It seems likely that, with the similarity in the mode of transferase action, the other ADPRTs will follow a similar reaction mechanism relying on the 'active site loop' or the *Gln/Glu*-X-E motif to differentiate between their substrates. The 'active site loop' or the *Gln/Glu*-X-E motif can also select the nature of the activated nucleophile that attacks the ADP-ribose$^+$ moiety from toxin to toxin. This is supported by the findings that actin, EF2, $G_{\alpha i}$, $G_{\alpha s}$ and the Rho GTPases all have target residues that are analogous to diphthamide and a nearby aspartate/glutamate residue similar to one of the key ADPRT binding residues seen in the eEF2–PAETA complex (Asp696 of EF2). This aspartate interacts with the 2-OH of ADP ribose in a manner similar to that of the catalytic glutamate of the ADPRT and this could be essential to the mechanism and form part of the mechanism that has not yet been captured in a crystallographic structure. This could be part of a conserved recognition site for all the ADPRT substrates. However, to confirm that the CT and α-3 toxins do have similar mechanisms, further structural studies with their substrates must be performed.

Despite this wealth of knowledge, there are still many questions that are still left unanswered and it will require further research and many more structures to answer them: (a) How do the other classes of ADPRT recognize their substrates? In particular, what determines the very tight specificity of the C3-like exoenzymes that differentiate between the Rho GTPases targeting only A-C? (b) Do other bacterial ADPRTs follow the same reaction pathway as that proposed for EF2-PAETA from recent biochemical, kinetic and structural studies? (c) How do the substrate recognition and substrate interactions differ between the 'active site loop' and 'α3- helix' proteins? (d) What role, if any, do the C3-like exo-enzymes play in bacterial pathogenesis?

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