NAD Binding Induces Conformational Changes in Rho ADP-ribosylating Clostridium botulinum C3 Exoenzyme*

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We have solved the crystal structures of Clostridium botulinum C3 exoenzyme free and complexed to NAD in the same crystal form, at 2.7 and 1.95 Å, respectively. The asymmetric unit contains four molecules, which, in the free form, share the same conformation. Upon NAD binding, C3 underwent various conformational changes, whose amplitudes were differentially limited in the four molecules of the crystal unit. A major rearrangement concerns the loop that contains the functionally important ARTT motif (ADP-ribosyltransferase toxin turn-turn). The ARTT loop undergoes an ample swinging motion to adopt a conformation that covers the nicotinamide moiety of NAD. In particular, Gln-212, which belongs to the ARTT motif, flips over from a solvent-exposed environment to a buried conformation in the NAD binding pocket. Mutational experiments showed that Gln-212 is neither involved in NAD binding nor in the NAD-glycohydrolase activity of C3, whereas it plays a critical role in the ADP-ribosyl transfer to the substrate Rho. We observed additional NAD-induced movements, including a crab-claw motion of a subdomain that closes the NAD binding pocket. The data emphasized a remarkable NAD-induced plasticity of the C3 binding pocket and suggest that the NAD-induced ARTT loop conformation may be favored by the C3-NAD complex to bind to the substrate Rho. Our structural observations, together with a number of mutational experiments suggest that the mechanisms of Rho ADP-ribosylation by C3-NAD may be more complex than initially anticipated.

Many bacterial toxins ADP-ribosylate nucleotide-binding proteins that are involved in essential cell functions (for review see Ref. 1). The molecular basis of their action consists of the binding of NAD, its glycohydrolysis into ADP-ribose and nicotinamide, and the transfer of the ADP-ribose moiety to a specific residue on the eukaryotic protein substrate. All these toxins share a highly conserved catalytic glutamate, which is critical for the NAD-glycohydrolase activity. Although they exhibit a similar global mode of action, ADP-ribosyltransferase toxins are quite distinct regarding their substrate and their pathophysiological properties. Thus, these toxins can be divided into four subfamilies: diphtheria-like toxins, cholera-like toxins, binary toxins and C3-like exoenzymes.

C3-like exoenzymes are distinct from other ADP-ribosyltransferase toxins in that they lack specific cell-surface binding and translocation components to facilitate their cell entry. Also, they are unique because of their high specificity for the small GTP-binding proteins RhoA, RhoB, and RhoC on an asparagine residue. This specificity makes them particularly useful to switch off selectively the cellular function of Rho proteins. Thus, C3-like exoenzymes are used to study the Rho-dependent processes, including cytoskeleton organization, endocytosis, phagocytosis, nucleus signaling, and regulation of gene transcription (for review see Ref. 2). However, the molecular basis of C3 action including NAD binding, its glycohydrolysis, Rho binding, and Rho ADP-ribosylation, is not well understood.

The structure of Clostridium botulinum C3 exoenzyme free of NAD was recently determined, revealing that C3-like exoenzymes possess the main features that characterize the NAD-binding site of other ADP-ribosyltransferase toxins (3). These authors have proposed that C3-like exoenzymes and binary toxins should be included in the same subfamily on the basis of structural and sequence similarities in their catalytic domain. A new motif of this subfamily has been described and termed the ARRT1 (ADP-ribosylating toxin turn-turn) motif (3). This motif of two short amino acid stretches (or turns) encompasses residues 207–210 and 211–214 and may be important for catalytic activity and substrate recognition (3, 4). The highly conserved catalytic glutamate (Glu-214) belongs to this motif. Another similarity with the C3-like exoenzymes and binary toxins is the absence of the “active site” loop that has been described for other ADP-ribosyltransferase toxins as a substrate recognition site (5, 6).

To approach the molecular basis of C3 action, we carried out a structural study of the C. botulinum C3 exoenzyme complexed to NAD. Crystals of C3 free of NAD were initially obtained with four independent molecules in the asymmetric unit and diffracted to 2.7 Å resolution. To prevent NAD hydrolysis during crystal growth, we briefly soaked these crystals in NAD solution. Hence, the structure of C3 complexed to NAD was determined at 1.95 Å resolution, preventing crystallographic bias in a structural comparison with the free form.

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The atomic coordinates and structure factors (code 1gce, r1gcesf, 1gcf, and r1gcfjf) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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The abbreviations used are: ARRT, ADP-ribosyl toxin turn-turn; GST, glutathione S-transferase; r.m.s.d., root mean square deviation; NMN, nicotinamide mono-nucleotide; PN, phosphate-nicotinamide.
These structures showed that upon NAD binding, C3 underwent various conformational changes whose amplitudes were differentially limited in the four molecules of the crystal unit. Our data emphasized a remarkable NAD-induced plasticity of the NAD binding pocket and suggest that a novel ARTT conformation may be privileged by the C3-NAD complex to bind to Rho.

EXPERIMENTAL PROCEDURES

Protein Expression—The mature form of C. botulinum C3 exoenzyme (residues 41–259) was subcloned into the pET-28a vector, expressed in the BL21 (DE3) strain of Escherichia coli, and purified to homogeneity in one step by cation exchange chromatography (CM-Sepharose Fast Flow). Mutants were constructed by site-directed mutagenesis with the pET-28a C3 wild-type as templates and the respective oligonucleotides using the Stratagene QuikChange kit according to the manufacturer’s instructions. The different C3 mutants (S174A, L177C, Q182A, R166E, and Q212A) were expressed and purified as the wild-type. RhoA was produced as a pure protein as previously described (7) using the pET-28a RhoA-F28N vector (gift of A. Hall) expressed in the XLI Blue strain of E. coli after cleavage of the GST moiety from the fusion protein RhoAGST with 3 units of thrombin for 18 h at 4 °C.

Crystallization—Crystals of wild-type and mutant C. botulinum C3 exoenzyme in solution were grown in sitting drops by the vapor diffusion method at co-crystallization to limit NAD hydrolysis during crystal growth. Consequently, the C3-L177C structure represents well the structure of models reported by Han et al. and 80 mM citric acid at pH 3. Seeding techniques were applied to NAD was directly refined starting from the coordinates of C3-free.

To measure ADP-riboinosyltransferase activity of C3 wild-type or its mutants, 0.1 nmol of the exoenzyme and 1 nmol of NAD enriched with 151P was incubated in 10 μL of phosphate buffer, 10 mM, pH 7.5, which was incubated for 2, 50, 100, and 120 min at 37 °C. 1 μL of each sample was then loaded on a cellulose MN 300 sheet (Polygram CL 300 PEI/UV 254, Machery-Nagel) and eluted with 100% ethanol:H2O:sodium acetate 1:x at pH 5.20:40:20. Radioactivity was detected and quantified with an imaging system (Storm 840, Molecular Dynamics). Results are representative of three independent experiments and were subjected to an analysis of variance (StatView).

To measure ADP-riboinosyltransferase activity of C3 wild-type or its mutants, 0.1 nmol of the exoenzyme diluted in 10 μL of Tris 50 mM, pH 7.5, containing 100 mM NaCl, 20 mM MgCl2, 10 mM dithiothreitol and 13 mM 151P-NAD was incubated for 1 h at 37 °C with 0.05% of RhoA. This mixture was then loaded on a 12% SDS polyacrylamide gel, and radioactive RhoA was directly detected and quantified on the dried gel by an imaging system (Storm 840, Molecular Dynamics)

NAD glycohydrolase and ADP-riboinosyltransferase Activity Measurements—To measure the NAD-glycohydrolase activity of C3 wild-type or its mutants, 0.1 nmol of the exoenzyme and 1 nmol of NAD enriched with 151P-NAD in 10 μL of phosphate buffer, 10 mM, pH 7.5, which were incubated for 2, 50, 100, and 120 min at 37 °C. 1 μL of each sample was then loaded on a cellulose MN 300 sheet (Polygram CL 300 PEI/UV 254, Machery-Nagel) and eluted with 100% ethanol:H2O:sodium acetate 1:x at pH 5.20:40:20. Radioactivity was detected and quantified with an imaging system (Storm 840, Molecular Dynamics). Results are representative of three independent experiments and were subjected to an analysis of variance (StatView).

Data collection and refinement statistics

| Data                          | C3-free | C3-NAD |
|-------------------------------|---------|--------|
| Soaking delay                 | 60 min  |        |
| Space group                   | C2      | C2     |
| Resolution (last shell) (Å)   | 2.0–2.7 | 2.5–1.95 |
| Unit cell (a, b, c) (Å)        | 109.0, 75.6, 123.5 | 103.9, 74.0, 119.7 |
| Measurement reflections       | 102.4   | 102.1  |
| Unique reflections            | 120624  | 157646 |
| Completeness (last shell) (%) | 96.6 (76.3) | 96.8 (62.0) |
| Mean R(fo/fo)                 | 9.9 (1.8) | 16.3 (2.6) |
| R(free) (%)                   | 7.2 (39.9) | 5.6 (27.3) |
| Average B-factor (Å2)         | 99.9     | 99.9   |
| Number proteins               | 5008/400 | 5008/400 |
| atoms/waters                  | 6467/43 | 6486/571 |
| R.m.s.d. bond (Å) (Angles) (%)| 0.012/1.7 | 0.027/1.8 |

RESULTS

Overall Description—The structures of C. botulinum C3 exoenzyme free (C3-free) and complexed to NAD (C3-NAD) were solved by molecular replacement at 2.7 and 1.95 Å resolution, respectively. Both structures were obtained in the same crystal form, which includes four independent molecules in the asymmetric unit, named hereafter and in the PDB coordinate names as A, B, C, and D. In the C3-free form, each of the four molecules adopts an overall structure that is similar to the three others or to the one previously described (3) (r.m.s.d. < 0.67 Å; Table II). The overall structure of the four NAD-bound C3 molecules is also conserved, and each of them is similar to its equivalent unbound form (r.m.s.d. < 0.88 Å; Table II). The overall structure of C3 is a mixed α/β fold with a β-sandwich core formed by perpendicular packing of a five-stranded mixed β-sheet (β1, β4, β8, β7, and β2) against a three-stranded antiparallel β-sheet (β3, β6, and β5). The three-stranded sheet is flanked by four consecu-
TABLE II

| r.m.s. deviation (Å) |
|---------------------|
| Molecule A | Molecule B | Molecule C | Molecule D |
| C3-free | | | |
| Molecule A | 0.55 | 0.67 | | |
| Molecule B | 0.48 | 0.67 | 0.50 | 0.29 |
| Molecule C | 0.56 | 0.67 | 0.57 | 0.28 |
| Molecule D | 0.57 | 0.67 | 0.57 | 0.28 |
| C3-NAD (1) | 0.48 | 0.67 | 0.57 | 0.28 |
| Molecule A | 0.74 | 0.67 | 0.66 | 0.57 |
| ARTT loop | 2.56 | 0.80 | 0.50 | 0.67 |
| Molecule B | 0.88 | 0.67 | | |
| Molecule C | 0.67 | 0.50 | 0.67 | 0.50 |
| Molecule D | 0.57 | 0.73 | 0.67 | 0.73 |

NAD Binding Induces C3 Exoenzyme Conformational Changes

The root mean square deviation was calculated on the main chain atoms of residues 45–245: (1). An average r.m.s.d. from the four C3 molecules present in the asymmetric unit of the C3-free structure (PDB accession code 1G24) was reported: (2). The r.m.s.d. reported for the molecule was calculated with omission of residues 204–212. The r.m.s.d. reported for the ARTT loop was calculated with residues 204–212.

The ARTT Loop Changes Its Conformation upon NAD Binding—We have made a detailed structural comparison of the ARTT loop between the unbound and NAD-bound forms of C3. Because the C3-free and C3-NAD structures have been solved in the same crystal form, this analysis is prevented from crystal-packing bias. The NAD-induced ARTT loop movement causes a swinging rearrangement of some of its side chains from a solvent-exposed environment to a buried conformation (Fig. 4). In particular, the side chain of Glu-212 makes a large switch (7 Å Cα-Cα atoms) toward the interior of the NAD-binding cleft, where it interacts with the O-2′-hydroxyl of the nicotinamide ribose. Also, but to a lesser extent, Ser-207 and

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Phe-209 move closer to NAD (Fig. 4, A and B). In this novel conformation, the ARTT loop and the three side chains covered the ribose-nicotinamide moiety in the NAD-binding site. The ribose-nicotinamide moiety is further buried by stacking with the aromatic side chain of Phe-183 from the PN loop (Fig. 4, A and B). All these residue movements lead to the formation of an adjusted pocket that surrounds the ribose-nicotinamide moiety in the NAD-binding site (Fig. 4 C). Gln-212 contributes to the formation of this pocket by forming a network of interactions (Fig. 4 B). It interacts with Gln-182 from the PN loop, which in turn lies along the aromatic ring of Phe-183 and with a water molecule that links Ser-207 and the catalytic Glu-214. This water molecule takes the same position as the Gly-211 amide from the ARTT loop, which has moved by 6 Å upon NAD binding (Fig. 4, A and B). The catalytic Glu-214 is maintained in the same position in the free and NAD-bound forms for two reasons. First, the interaction between Glu-214 and Gly-211 in the free form is no longer possible in the NAD form due to the glycine movement but is replaced by indirect interactions with Gln-212 and Ser-207 through a water molecule. Second, the interaction of Glu-214 with Ser-174 from the STS motif, which forms the bottom of the site, is preserved. These two sets of interactions lock Glu-214, which thus interacts with the O-2'-hydroxyl of the nicotinamide ribose (Fig. 4 B), in agreement with the proposed mechanism of catalysis (18, 19). Therefore, upon NAD binding, the ARTT loop undergoes a major move-
NAD Binding Induces C3 Exoenzyme Conformational Changes

Interactions between NAD and C3

| NAD Residue Region | C3 (molecule A) Region | Distance (Å) |
|-------------------|------------------------|--------------|
| Nicotinamide-O-7  | Gly-129-N               | 3.0          |
| Nicotinamide-O-7  | Gly-129-O               | 2.9          |
| Ribose-O-2'       | Glu-214-OE1             | 2.9          |
| Ribose-O-2'       | Gln-212-NE2             | 3.3          |
| Ribose-O-3'       | Thr-89-O                | 2.7          |
| PN-O-1            | Arg-128-NH1             | 3.3          |
| PN-O-2            | Arg-186-NH1             | 3.0          |
| PA-O-1            | Arg-128-NH1             | 3.3          |
| PA-O-2            | Arg-128-NH2             | 2.8          |
| Ribose-O-3'       | Asp-87-ND2              | 2.8          |
| Ribose-O-2'       | Asp-131-OD2             | 2.8          |
| Ribose-O-2'       | Asp-131-N               | 2.8          |
| Ribose-O-2'       | Asp-130-OD1             | 2.7          |
| Adenine-N-6       | Glu-169-OE1             | 2.9          |
| Adenine ring      | Arg-91-side chain       | 3.4          |

Other Conformational Changes—Another important change that occurs in C3 upon NAD binding is a “crab-claw” movement, which closes and opens the ADP moiety-binding site. It concerns a large subdomain consisting of three strands and a helix (β8-β7-β2 and α5), which undergoes a hinge movement of ~9° with respect to the rest of the molecule (Fig. 5). Structural superimposition on helices α2, α3, and α4 between the unbound and NAD-bound forms for each of the four C3 molecules in the asymmetric unit showed that in molecules A, B, and C this movement closes the ADP moiety-binding site by 5°, 8°, and 4°, respectively. The major consequence is the shift of the C-terminal extremity of the strand β1 by ~1.7 Å toward the ADP moiety of NAD and consequently the formation of interactions of residues Asp-130 and Asp-131 with the hydroxyl of the adenine ribose. In contrast in molecule D, where only ADP is bound, the ADP moiety-binding site is more open by 3° than when empty (Fig. 5). The opening of the ADP moiety-binding site together with the weak electron density observed for ADP (Fig. 5) suggests that the co-substrate is less stabilized in this conformation. Therefore, the crab-claw closure may correlate with NAD stabilization, whereas the opening might reflect the local conformation associated with product release. This crab-claw movement is independent of the ARTT loop conformation.

Fig. 3. Electron density and B-factors of the co-substrates. A, NAD bound to molecule A, B, NAD bound to molecule B, C, ADP and ribose-nicotinamide bound to molecule C, D, ADP bound to molecule D. The co-substrates are colored based on B-factor values from blue to red ranging from 20–90 Å², respectively. The Fo – Fc electron density map at 1.95 Å resolution with the co-substrate omitted from the calculation is shown in light gray and contoured at the 2σ level. E, NAD model in which nitrogen, oxygen, and phosphorus atoms are colored in blue, red, and orange, respectively. The NAD-glycohydrolyzable bond (N-1–C-1') is shown in yellow with the nicotinamide moiety on its right and the ADP-ribose moiety on its left.
because the closures observed in molecules A and B are similar, although they display different ARTT loop conformations.

A “serine-threonine-serine” sequence, called the “STS motif” (strand β3; Fig. 1) stabilizes the NAD-binding cleft by connecting the two perpendicular β-strands, which form the bottom of the cleft. The first serine of the STS motif, Ser-174, which is important for catalytic function in other toxins (20, 21), adopts two different orientations in our structure. In the first orienta-
tion, Ser-174 interacts with the carboxylate of the catalytic Glu-214 and the hydroxyl of Tyr-79 (Fig. 4B), as observed in the binary toxin, VIP2, which suggests a role in properly positioning the catalytic glutamate (18). In the second orientation, Ser-174 interacts with the phosphate group of the NMN moiety, close to the NAD cleavage observed in molecule C. In this position, it may play a role in NAD hydrolysis and could be responsible for the hydrolysis observed in our crystal structure.

**Mutational Experiments**—Structural data have shown that Gln-212 and to a lesser extent Gln-182 are involved in the conformational changes induced by NAD binding. We therefore probed their contribution to NAD binding and to both NAD-glycohydrolase and ADP-ribosyltransferase activities by substituting them individually into alanine. Plasmon resonance experiments showed that NAD binds to the Q182A and Q212A mutants with approximately the same and 2-fold lower affinity as compared with the wild-type enzyme, respectively (Table IV). These data show that neither of these two residues plays a major role in the binding energy of NAD to C3. The NAD-glycohydrolase activity of these mutants was probed by following the \([\text{32P}]\text{NAD}\) hydrolysis by chromatography. Fig. 6a shows that the Q212A mutant has virtually the same activity as the wild-type enzyme and that the Q182A mutant even has a slightly increased activity. Neither of these two residues is a major contributor to the NAD-glycohydrolase activity of C3. A different result was obtained for the ADP-ribosyltransferase activity as deduced from a qualitative monitoring of the transfer of the \([\text{32P}]\text{ADP-ribose}\) moiety to RhoA by electrophoresis. Thus, we found that the Q212A mutant was virtually inactive, whereas the Q182A mutant was not affected in its capacity to ADP-ribosylate Rho (Fig. 6b). Clearly, Gln-212 plays no major role in NAD hydrolysis but is critical for the ADP-ribosyl transfer to RhoA, whereas Gln-182 does not have a predominant function in either activity.

The contribution of Arg-186 from the PN loop was also probed by mutagenesis. The reason for this experiment was that this residue interacts with the phosphate group of the NMN moiety of NAD and is conserved in most C3-like exoenzymes and binary toxins. To evaluate the role of Arg-186 in NAD binding and ADP-ribosyltransferase activity, we generated the mutant R186E, with a charge reversal at this position. This mutant was unable to bind NAD (Table IV) and hence had no catalytic activity (Fig. 6b). Therefore, Arg-186 is involved in the interaction with NAD.

Our structural observations have revealed that in the presence of NAD, Ser-174 from the STS motif can adopt two different orientations; one where it interacts with the catalytic Glu-214 and another where it interacts with the phosphate group of the NMN moiety of NAD. To investigate the role of Ser-174 in the catalytic mechanism, we prepared the S174A mutant and tested its ADP-ribosyltransferase activity. This mutant retains the wild-type ADP-ribosyltransferase activity (Fig. 6b), suggesting that Ser-174 plays no major role in *C. botulinum* C3 exoenzyme activity.

**DISCUSSION**

This structural study has shown that binding of NAD to the C3 exoenzyme has induced various conformational changes, differentially limited by the crystal-packing environment. Two major regions of the toxin are involved in these movements. The first one concerns the functionally important ARTT loop (14), which undergoes an ample motion that occurs only in the absence of local crystal-packing constraints. Such a movement is not unique to C3 because upon NAD analogs binding to *P. aeruginosa* exotoxin A the region equivalent to the ARTT loop also undergoes a conformational change (22). The NAD-induced ARTT loop movement observed in C3 causes a large swinging rearrangement of some of its side chains from a solvent-exposed environment to a buried conformation in the NAD-binding site. In this latter conformation, these side chains cover the nicotinamide ribose moiety and hence contribute to the formation of a network of interactions that also involves residues from the adjacent PN loop. Surprisingly, mutational experiments on Gln-212 from the ARTT loop and Gln-182 from the PN loop that are both involved in this network of interaction revealed that these residues make no predominant energetic contributions to NAD binding. Therefore, the NAD-in-
duced ARTT loop conformational change may not be required for NAD to bind to C3. A second major NAD-induced movement of C3 concerns a large subdomain that undergoes a claw-claw-like motion and thus closes the binding site of the AMP moiety of NAD. As a result of this movement, several residues of this subdomain establish direct contacts with NAD, including Arg-128, Gly-129, and the two aspartate 130 and 131 residues. The equivalent subdomain of the binary toxin of B. cereus vegetative insecticidal protein VIP2 also undergoes such a movement (18), suggesting that this motion may be a common means for ADP-ribosyltransferase toxins to bind NAD. Additional though less ample changes were noticed in the vicinity of the NAD binding pocket as a result of NAD binding. They concern residues of the PN loop, which include Gln-182 and Phe-183, whose side chains tend to stack above the nicotineamide ring, and Arg-186 whose side chain interacts with the phosphate group of the NMN moiety of NAD. Therefore, upon NAD binding several regions of the NAD binding pocket undergo structural rearrangements of variable amplitudes.

What could be the biological implications, if any, of these NAD-induced conformational changes in C3? Binding of NAD to C3 is a prerequisite for the Rho ADP-ribosylation reaction to occur (23). Also, evidence has been accumulated to indicate that the ARTT loop, and in particular Gln-212 are critical factors of the ADP-ribosylation process (Refs. 4, 14 and this study). We found that the ARTT loop may exist in two distinct conformations when NAD is bound to C3. In one of them, the loop adopts a solvent-exposed side chain conformation as a result of local crystal-packing constraints that prevent it from moving freely. In the other, the loop, which has undergone a large swinging movement upon NAD binding, adopts a side-chain-buried conformation. Therefore, we propose that this latter conformation, which is unconstrained by crystal-packing contacts may predominantly reflect the inherent conformational trend of the ARTT loop in the presence of NAD. This might be a privileged form for the C3-NAD complex to bind to Rho. In this structure Gln-212 is buried in the NAD binding pocket, and hence it is inaccessible to Rho, a situation that is not incompatible with the previous observation that this residue is not an energetically important contributor of Rho binding (4).

The role of Gln-212 in the ADP-ribosyl transfer to Rho (4, 24) is presumably specific because this residue neither contributes to NAD binding (this work and Ref. 4) nor to Rho binding (4, 24), nor to NAD hydrolysis (this work and Ref. 4). On the basis of structural comparison between binary toxins and C3-like exoenzymes, a model has been proposed that suggests that Gln-212 is solvent-exposed to interact specifically at position Rho Asn-41 for ADP-ribosylation. As discussed above, our data suggest a plausible Rho-binding conformation of the C3-NAD complex in which Gln-212 is buried and, hence, unlikely to be directly accessible to Rho Asn-41. Would the binding of Rho be able to trigger a further conformational change of the ARTT loop to make Gln-212 switching from a buried to a solvent-exposed conformation? This is not inconceivable because in the presence of NAD and a particular crystal-packing environment (Fig. 1B), the side chain of Gln-212 exists in an exposed conformation.

We found it intriguing that Ser-174 interacts with the catalytic Glu-214 in both the unbound and NAD-bound molecule A, while it changes its orientation in the NAD-bound molecule B, where it makes contact with the phosphate group of the NMN moiety of NAD. However, the mutant S174A showed no difference in ADP-ribosyltransferase activity, discarding the possibility that Ser-174 may be a major actor in this activity, as also recently shown for the Staphylococcus aureus C3 exoenzyme (4). Along similar lines of thought, we were intrigued by the slight movement undergone by Arg-186, which switched from a flexible state to a well defined interacting conformation with the same phosphate group of the NMN moiety. We found that reversal of the charge of Arg-186 made C3 unable to bind to NAD and hence to express an ADP-ribosyltransferase activity. Because Arg-186 is exclusively conserved in most C3-like exoenzymes and binary toxins, we suggest that this residue is a functionally important feature of most C3-like exoenzymes and binary toxins.

Altogether, our observations suggest that the mechanism of Rho ADP-ribosylation by C3 may be more complex than originally anticipated. The plasticity of the critical ARTT loop evidenced in this work may have to be taken into account to understand this mechanism. We propose that the C3 activity may require several subtle rearrangements of the ARTT loop involving at least three steps: NAD binding, Rho binding, and Rho ADP-ribosylation. A similar multistep mechanism is also suggested by a mutational study in which the non-substrate Rac was progressively transformed into a C3-binding form and then into a true C3 substrate form (25).

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