The Crystal Structure of C3stau2 from Staphylococcus aureus and Its Complex with NAD*

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The C3stau2 exoenzyme from Staphylococcus aureus is a C3-like ADP-ribosyltransferase that ADP-ribosylates not only RhoA-C but also RhoE/Rnd3. In this study we have crystallized and determined the structure of C3stau2 in both its native form and in complex with NAD at 1.68- and 2.02-Å resolutions, respectively. The topology of C3stau2 is similar to that of C3bot1 from Clostridium botulinum (with which it shares 35% amino acid sequence identity) with the addition of two extra helices after strand β1. The native structure also features a novel orientation of the catalytic ARTT loop, which approximates the conformation seen for the “NAD bound” form of C3bot1. C3stau2 orients NAD similarly to C3bot1, and on binding NAD, C3stau2 undergoes a clasp motion and a rearrangement of the phosphate-nicotinamide binding loop, enclosing the NAD in the binding site. Comparison of these structures with those of C3bot1 and related toxins reveals a degree of divergence in the interactions with the adenine moiety among the ADP-ribosylating toxins that contrasts with the more conserved interactions with the nicotinamide. Comparison with C3bot1 gives some insight into the different protein substrate specificities of these enzymes.

The family of C3 ADP-ribosyltransferases is a subgroup of the ADP-ribosyltransferase toxins that also include the A-B toxins such as diphtheria toxin and cholera toxin and the binary toxins, which include C2 from Clostridium botulinum, the vegetative insecticidal protein (VIP) from Bacillus cereus, and the Iota toxin from Clostridium perfringens. The targets for the C3 ADP-ribosyltransferases are mammalian Rho GTPases, but they are novel among the ADP-ribosylating toxins in that they lack a cell binding or translocation domain to allow entry into cells, and hence, their role in disease is not yet clear. However, the best-characterized member of this family, the C3 exoenzyme from C. botulinum, C3bot1, has long been used to research the function of the small mammalian GTPases. This is due to its ability to specifically ADP-ribosylate and, therefore, inactivate RhoA, -B, and -C (1) but not the related proteins Rac and Cdc42 (2–4). C3bot1 has been described as the prototype for this family of ADP-ribosyltransferases, which also includes C3 from Clostridium limosum (C3lim) (4), B. cereus (C3cer) (5), and the epidermal differentiation inhibitor (EDIN) (6) from Staphylococcus aureus.

The two isoforms of C3 from C. botulinum, known as C3bot1 (7, 8) and C3bot2 (9), have so far been assumed to represent the whole family and have attracted the most research. Recently, however, the existence of a subgroup of the family has emerged with the discovery of two proteins from S. aureus named C3stau2 (or EDIN B) (10–12) and C3stau3 (or EDIN C) (13). Whereas the C3s from C. botulinum and C. limosum have 63% sequence identity (4), the C3stau exoenzymes have only 35% sequence identity with the clostridial C3s, although they are 65% identical to each other (Fig. 1). Interestingly, C3stau2, and very recently, EDIN (C3stau1) have been shown to have substrate specificities different from that of C3bot1, ribosylating the related GTPases RhoE and Rnd3 as well as RhoA-C (10, 14).

Inactivation of RhoA-C after C3-mediated ADP-ribosylation at Asn-41 impacts on cell functions in various ways, reflecting the many roles of Rho in mammalian cells. Through the use of C3bot as a research tool it has been shown that Rho is involved in many cell activities through its regulation of the cytoskeleton and transcription. These include cell cycle progression, chemotaxis, cell transformation, and apoptosis (15). RhoE and Rnd3 are isoforms, identical except for a 15-residue N-terminal extension on Rnd3, that are antagonistic to RhoA (16, 17). Unusually, they bind GTP but lack GTPase activity. This renders them constitutively active (18), and they are, therefore, speculated to reduce RhoA activity by sequestering the exchange factors required to activate it (10). Surprisingly, although parallel C3stau2-mediated ribosylation of RhoA and RhoA antagonists might be presumed to produce a different cell morphology to that produced by C3bot1 exposure, both proteins produce the same cell rounding effect in fibroblasts (10).

The role of the clostridial C3 exoenzymes in pathogenesis is uncertain due to the lack of an obvious cell entry mechanism. The C3stau exoenzymes, however, may have a more obvious role due to the ability of S. aureus to invade host cells, circumventing the need for a binding or translocation domain. It has been shown that EDIN and C3bot1 can prevent wound healing in vivo, indicating a possible role for the C3stau exoenzymes in S. aureus infection (19). Additionally, preliminary findings show that some clinical isolates produce C3stau exoenzymes, C3stau2 in particular (20), and another study reports two in-
EDTA electrophoresis buffer, and a principal band of ~750 base pairs was excised from the gel. The band was extracted from the gel using QIAEX II gel extraction resin (Qiagen) and subcloned into the sequencing vector PCR4.0 TOPO (Invitrogen) according to the manufacturer’s instructions. The clone was sequenced and showed two point mutations in the regions of primers C3sBamXa_for and C3s10.rev. These were corrected by amplification with these primers followed by extension to give the full-length clone and re-amplification with the flanking primers. Cloning and sequence verification were carried out as described above.

**Protein Expression and Purification**—For expression of the C3stau2 fragment, a modified malE fusion vector was generated to ensure that the gene could not be disseminated to other bacteria. The Apal-HindIII fragment from the expression vector pMALc2x (New England Biolabs) was isolated and subcloned into the vector pBC SK+ (Stratagene) to generate the vector pBCmalE. The construction of the vector was confirmed by restriction digest and sequencing. A BamHI fragment containing the C3stau2-coding sequence was subcloned into BamHI-digested pBCmalE, and the clone was verified. For expression, the clone E. coli TB1 pBCmalE C3stau2 was grown overnight at 30 °C in Terrific Broth supplemented with 35 μg/ml chloramphenicol and 0.5% (w/v) glucose. The overnight culture was diluted 1:10 in fresh media and grown for 4 h (A600 ~ 3.9). The culture was induced with isopropyl-1-thio-β-D-galactopyranoside at a final concentration of 500 μM and grown for a further 2 h 30 min at 25 °C before harvest. The cells were collected by centrifugation and resuspended in 20 mM MES/NaOH, pH 5.8. The cells were lysed by sonication, and the cell lysate was collected by centrifugation.

The C3stau2-MBP fusion protein was initially purified by cation exchange chromatography. The protein bound to a SP-Sepharose column equilibrated with 20 mM MES/NaOH, pH 5.8, and was eluted at an ascending NaCl gradient as a single peak. The fractions corresponding to the peak were pooled, and 150 units of Factor Xa were added per half-liter of cells to cleave the fusion protein. During cleavage, the protein was dialyzed for 24 h at room temperature against a buffer containing 20 mM Na-HEPES, pH 7.3, 50 mM NaCl, and 5 mM CaCl2.

**EXPERIMENTAL PROCEDURES**

**Cloning and Expression**—A synthetic gene encoding C3stau2 was synthesized using codon bias optimized for expression in *E. coli*. A series of overlapping oligonucleotides was synthesized (Sigma-Genosys) (Table I). In addition to the structural oligonucleotides, flanking primers were designed to introduce a 5′ and a 3′ BamHI site and position of helix α3 that may govern target protein specificity.

**Crystal Structure of C3stau2 from Staphylococcus aureus**

**FIG. 1.** Sequence alignment of C3stau2, EDIN (C3stau1), C3bot1, C3lim, and VIP2 as computed by T-Coffee (35). Secondary structural elements for C3stau2 are shown above the alignment. Residues conserved throughout are shown in red, and those conserved only in the C3 toxins are shown in blue. The FN loop is marked in gray between β3 and β4, and the ARTT loop is in gray between β5 and β8. Residues numbers are given as published for the structures of C3bot1 (22) and VIP2 (24). C3stau2, EDIN, and C3lim numbering starts at the first residue of the mature polypeptide.
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The cleaved C3stau2 bound to the SP-Sepharose column equilibrated in 20 mM Na-HEPES, pH 7.3, 50 mM NaCl and was eluted again on an ascending salt gradient. Final purification and de-salting was achieved by gel filtration. The protein was concentrated in a 10-kDa cut-off centrifugal concentrator (Amicon) and applied to a Superdex 200 gel filtration column (Amersham Biosciences) equilibrated in 20 mM Na-HEPES, pH 7.3, 50 mM NaCl. The purified protein was finally concentrated for gel filtration and stored in 20 mM Na-HEPES, pH 7.3, 50 mM NaCl at −70 °C. Purity was greater than 95% as judged by SDS-PAGE.

The specific ADP-ribosylating activity of the preparation was 0.19 mol/mol enzyme/min as measured by the labeling of 19S RhoA by 11 μM C3stau2 in the presence of 20 μM [α-32P]NAD at 37 °C. NAD was added to the model at a ratio of 1.0 to C3stau2 (8.75–10.25 mg/ml) was mixed with 1 μl of well solution (100 mM sodium cacodylate, pH 6.4–6.6, 29–30% PEG (polyethylene glycol) 8000). Orthorhombic crystals grew within 1–3 weeks. Seeding produced larger numbers of crystals, which grew within 3 days and were suitable for cryo-diffraction.

Structure Determination—Native crystals were soaked in cryoprotectant (0.1 x sodium cacodylate, pH 6.5, 30% PEG 8000, 20% glycerol) and cryo-cooled to 100 K. The crystals diffracted to 1.68 Å resolution, increasing to 1.68 Å R-factor of 17.0% and an R-free value of 23.7%. The N and C termini of well solution (100 mM sodium cacodylate, pH 10.25 mg/ml) showed clear differences against the native C3stau structure.

The behavior of the R-factor for 56% and a correlation coefficient of 31.4%. Initial refinement was carried out using CNS version 1.0 (29) at 2.6 Å resolution, increasing to 1.68 Å. The behavior of the R-free value (30) was monitored throughout. Insertion of water molecules and final refinement were performed in SHELX (31). All the water molecules had peak heights above 3 σ in the Fc – F map and temperature factors less than 40 Å². The program O (32) was used to rebuild the model and visualize the maps. The final model contains 1660 protein atoms and 284 water molecules and has a crystallographic R-factor of 17.0% and an R-free value of 23.7%. The N and C termini were clearly visible in the structure, but residues 197–199 were slightly disordered and have been modeled on the basis of weak density. The extremities of side chains of 85, 94, 116, 170, and 200 have been disordered and have been modeled on the basis of weak density. The catalytic glutamate on turn 2, there is a conserved glutamate thought to be essential for C3stau ADP-ribosylation. As well as inserts termed for C3bot1, the C3stau2 ARTT Loop shows the same topology as the native form of C3stau2 with a C α atoms of 1.6 Å. Here, the main chain oxygen of residues Asn-199 and Lys-200 are bonded to the main chain nitrogen of Tyr-114 and the side-chain nitrogen of Gln-133, respectively.

NAD was added to the second model after initial refinement against the native C3stau structure showed clear difference density at 2σ level. The complex form has the same topology as the native form of C3stau2 with a C α r.m.s. deviation of 0.83 Å. Analysis of the Ramachandran (ϕ-ψ) plot showed that all residues lie in the allowed regions for both structures.

The ARTT Loop—The bacterial ADP-ribosylating toxins all share a conserved glutamate that is essential for ADP-ribosylation. In C3stau2, this residue (Glu-180) is located between strands β5 and β6 at the end of the second turn of what has been termed for C3bot1, the “ADP-ribosylating turn turn” (or ARTT) motif. The ARTT loop has been shown to be essential for binding the NAD and positioning the C3-NAD-Rho complex for ADP-ribosylation.

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RESULTS AND DISCUSSION

Overall Topology—The overall structure of C3stau2 is similar to that of C3bot1, i.e. a mixed α/β fold with a β-sandwich core (Fig. 2A) (22, 23). Structural alignment of C3stau2 with C3bot1 (Fig. 2B) shows that the main differences are restricted to the region of helices α5 and α6 (residues 92–126) in C3stau2, which corresponds to the loop between β1 and α5 in C3bot1 (residues 135–162) (Fig. 1). This region in C3stau2 includes seven extra residues that contribute to two extra helices comprising residues 99–108 (α5) and 115–125 (α6). This extended variable region is linked by main chain hydrogen bonding to the loop between strands β7 and β8 (residues 197–202), which also deviates from its counterpart in C3bot1 (r.m.s. deviation of Ca atoms is 1.6 Å). Here, the main chain oxygens of residues Asn-199 and Lys-200 are bonded to the main chain nitrogen of Tyr-114 and the side-chain nitrogen of Gln-133, respectively.

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ternary C3-NAD-Rho complex on turn 2 and a conserved tyrosine (or phenylalanine) on turn 1 necessary for Rho binding (11).

Despite the conserved role of the ARTT loop in ADP-ribosylation, the loop is orientated differently in C3stau2 and C3bot1. In the native structure of C3stau2, the backbone of the first turn of the ARTT loop is shifted by up to 5.1 Å compared with the backbone of the same turn in C3bot1 (Fig. 3), although this does not affect the conserved position of Glu-180. The three C3-like exoenzymes from S. aureus are unusual among the C3-like and binary toxins discovered so far in that the loops immediately before their ARTT loops contain an extra two residues. These two residues appear to be responsible for positioning the ARTT loop in a conformation identical not to that of the native C3bot1 structure but to that of the C3bot1-NAD complex, the NAD-bound conformation. The loop is well ordered with B-factors (mean = 10.2 Å² over 8 atoms) below average (15.3 Å²) for the structure and appears optimally placed for ribosylation.

It is not surprising then that upon binding of NAD, the ARTT loop undergoes just a slight conformational change (Fig. 3). The r.m.s. deviation for the ARTT loop between the native and the NAD-complexed structures over 8 Cα atoms is 0.1 Å.

As expected, Glu-180 can be seen hydrogen bonding to the nicotinamide ribose nitrogen, NO2' (Fig. 4), an interaction for which it does not need to move. In C3stau2, the position of Glu-180 is not stabilized by hydrogen bonding to the phosphate-nicotinamide (PN) loop as seen in C3bot1. However, Glu-180 does interact with Ser-138, the first serine in the ST motif (also known as the STS motif), as seen in C3bot1. The YX STX motif has been identified as a conserved motif across the ADP-ribosyltransferase toxins, and in the case of the C3 exoenzymes from S. aureus, this motif has the sequence YS STQ. This motif is not surprising then that upon binding of NAD, the ARTT loop undergoes just a slight conformational change (Fig. 3). The r.m.s. deviation for the ARTT loop between the native and the NAD-complexed structures over 8 Cα atoms is 0.1 Å.

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| Form of C3stau2 | Native | NAD complex |
|----------------|--------|-------------|
| Space group    | P2₁,2₁ | P2₁,2₁      |
| Unit cell dimensions (Å) | 1.89 | 2.02 |
| Measured reflections | 409,845 | 327,780 |
| Unique reflections | 22,619 | 14,088 |
| Complement (%) (last shell)^a | 99.4 (97.5) | 96.9 (96.6) |
| Mean R(ref) (last shell)^b | 20.0 (5.3) | 15.8 (4.1) |
| R_sym (%)^c (last shell) | 8.5 (29.5) | 8.8 (34.1) |
| Refinement | 17.0 | 21.4 |
| R_atom (%) | 23.7 | 26.9 |
| Number of protein atoms | 1,660 | 1,660 |
| Number of solvent atoms | 284 | 154 |
| Deviation from ideality (r.m.s.) | 15.3 | 24.5 |
| Bond lengths (Å) | 0.004 | 0.006 |
| Bond angles (°) | 1.3 | 1.3 |
| Average B-factors (Å²) | 15.3 | 24.5 |
| Protein molecules | 19.0 | 27.3 |
| Solvent molecules | 31.1 |   |
| Ligand atoms |   |   |

^a Last shell is 1.74–1.68 and 2.09–2.02 Å for native and NAD complex, respectively.

^b R_sym = Σ [h Fo(h) − Fc(h)]²/Σ Fo(h), where I(h) and F(h) are the observed and calculated structure factor amplitudes of reflection h, respectively.

^c R_fcrystal (last shell) is equal to R_sym for a randomly selected 850–950 reflections not used in the refinement (30).

Fig. 2. A, overall structure of C3stau2. Schematic representation in which helices, strands, and main chain termini are labeled. B, superposition of C3stau2 and C3bot1. C3stau2 is shown in green, and C3bot1 (22) is shown in blue. Alignment of the two structures was carried out using LSQ routines within O (32).

Fig. 3. A, superposition of the ARTT loops of C3bot1 and C3stau2. C3stau2 is in green, and C3bot1 is in blue. Residues from C3stau2 are prefixed s, and those from C3bot1 are prefixed b. B, superposition of the ARTT loops of the C3bot1-NAD complex and C3stau2. The C3stau2 ARTT loop rests in the NAD-bound conformation.
Fig. 4. Movement of residues involved in binding NAD. The native C3stau2 is shown in green, and the C3stau2-NAD complex is shown in pink with the NAD in blue. The large domain movements involved in binding NAD have moved residues Arg-85, Leu-86, Arg-150, Asn-88, Asp-90, Arg-48, and Glu-133 within binding distance of the NAD. Glu-180, Thr-37, and Gln-140 also bind NAD. For clarity, the interactions between the main chain nitrogens of Leu-86 and Asn-88 and the NO7 and AO2' atoms of the NAD, respectively, are not shown.

Fig. 5. Superposition of native C3stau2 and the C3stau2-NAD complex in two orientations. Native C3stau is in grey, and the domains that have moved to bind NAD in the complex are shown in pink. The PN binding loop is shown in green, and NAD is in blue. The first orientation (left) shows the change in the conformation of the PN binding loop most clearly. The second orientation (right) best shows the clasping of the domains to enclose the NAD in the binding site. The inset shows how the change in orientation of the PN binding loop allows Arg-150 to bind to the NAD.

C3bot1, Ser-174 (23), and C3stau2, Ser 138 (11), has very little effect on NAD binding or catalysis. Gln-140, however, the last C3stau2 residue in this motif, is positioned to hydrogen bond to the NO7 atom of NAD. These data may suggest that the role of this motif may vary between ADP-ribosyltransferases depending upon the contribution of the other residues in the NAD binding pocket to transition state stabilization.

The NAD-bound conformation of the native ARTT loop also has implications for Gln-178. This residue and its homologs in C3bot1 and the C. difficile Iota toxin have been shown by mutational analysis to have a role in ADP-ribosylation but not in isolated NAD or Rho binding (11, 23, 25, 38). This key residue, which is thought to stabilize the C3-NAD-Rho ternary complex, points toward the NAD binding cleft in a position identical to that of its equivalent residue in the C3bot1-NAD complex, Gln-212 (Fig. 3). Gln-178 does not move on NAD binding or hydrogen bond to the NAD confirming its nonessential role in NAD binding.

The other important residue at the end of turn 1 of the ARTT loop is Tyr-175. This tyrosine residue and its homolog in C3bot1, Phe-209, are the only residues so far implicated in RhoA binding. The solvent exposure of C3bot1 Phe-209 and its location near to the active site residues suggested a role in binding to a hydrophobic patch on RhoA (22), and the necessity of C3stau2 Tyr-175 for RhoA binding has been shown by mutational analysis (11). Once again, the position of this residue in native C3stau2 resembles that of its counterpart in NAD-complexed C3bot1 and does not move on NAD binding.

These results lend support to one obvious conclusion. The resting position of the ARTT loop in different C3-like exoenzymes can resemble to varying extents the conformation observed in the NAD-containing complex. The loop may of course rearrange further on ternary complex formation or during catalysis.

The PN Binding Loop—The structure of C3bot1 in complex with NAD identified a further loop between strands β3 and β4 comprising residues 141–150 in C3stau2, termed the PN loop (23). This loop is so-called as it appears to be involved in binding the nicotinamide and the nicotinamide-phosphate moieties of NAD through provision of a hydrophobic pocket. This loop was noted to alter its shape dramatically on the binding of NAD to C3bot1 (23). Interestingly, in C3stau2, this short loop also appears in a conformation different from that found in native C3bot1. In C3stau2, the loop appears more extended (Fig. 2), with an r.m.s. deviation of 2.5 Å over 10 Ca atoms when compared with C3bot1. Although this loop contains 2
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Potential hydrogen bonding interactions for C3stau2 and VIP2 were calculated using HBPLUS (40) with a distance cut-off of 3.3 Å and D-H-A angles >90°. Interactions for C3bot1 and the iota toxin were taken from the crystal structure papers (23, 25). The nicotinamide interactions are of the π-π type. The adenine ring interactions are of the π-π type in VIP2 but are van der Waals interactions in C3bot1.

### Table III

| NAD       | C3stau2 Distance | C3bot1 Distance | Iota toxin Distance | VIP2 Distance |
|-----------|------------------|-----------------|---------------------|--------------|
| Nicotinamide |                  |                 |                     |              |
| NO7       | Leu-86-N         | 3.0             | Phe-183 ring Gly-129-N | 3.0 | Arg-296-N | 2.7 | Trp-350-N | 2.6 |
| Gln-140-NE2 | 3.1             |                 |                     |              |
| NN7       | Leu-86-O         | 3.2             | Gly-129-O           | 2.9 | Arg-296-O | 2.9 | Trp-350-O | 3.0 |
| NO2       | Glu-180-EO1      | 2.5             | Glu-214-EO1         | 2.9 | Glu-380-EO1 | 3.1 | Glu-428-EO1 | 2.8 |
| NO3       | Gln-215-NE2      | 3.3             | Glu-301-EO2         | 3.3 | Arg-128-NH1 | 3.3 | Arg-352-NH1 | 2.8 |
| NO1       | Thr-37-O         | 2.9             | Thr-80-O            | 2.7 | Arg-128-NH1 | 3.3 | Arg-352-NH1 | 2.6 |
| NO2       | Arg-150-NH2      | 2.8             | Arg-186-NH1         | 3.0 | Arg-352-NH2 | 2.6 |                     |     |
| NO1       | Arg-150-NH1      | 3.1             |                     |              |
| AO1       | Arg-85-NH2       | 3.2             | Arg-128-NH1         | 3.3 | Arg-295-NH1 | 3.3 |                     |     |
| AO2       | Arg-48-NH1       | 2.9             | Arg-128-NH2         | 2.8 |                     |     |                     |     |
| AO3       | Asn-88-NE2       | 3.0             | Asp-131-OD2         | 2.8 |                     |     |                     |     |
| AO2       | Asn-88-N         | 2.7             | Asp-131-N           | 2.8 | Gln-300-NE2 | 3.1 |                     |     |
| Asn-90-OD2 | 2.4             |                 | Asp-130-OD1         | 2.7 |                     |     |                     |     |
| AN6       | Glu-133-EO1      | 2.9             | Glu-169-EO1         | 2.9 | Asn-335-OD1 | 3.2 |                     |     |
| AN7       | Arg-91 side chain |               |                     |              |

### Table III

| Adenine | Arg-315 side chain |
|---------|-------------------|

more alanines than in C3bot1, making a total of 3 alanines and 2 glycines, the mean B-factor for this loop (16.1 Å²) is not significantly higher than the mean for the whole molecule (15.3 Å²). The loop also makes three crystal contacts with symmetry-related molecules which may contribute to its stability in the crystal structure. It is, therefore, possible that this loop is more flexible in solution before NAD binding.

Fig. 5 shows that in contrast to the ARTT loop, the PN binding loop moves toward NAD on binding with a change in position corresponding to an r.m.s. deviation of 1.7 Å over 10 Ca atoms. C3stau2 does not have a residue equivalent to Phe-183 in C3bot1, which stacks with the adenine ring but provides a hydrophobic pocket of alanines, glycines, and Leu-148. The result of this conformational change is that Arg-150 is moved by ~1.0 Å closer to the NAD, allowing binding with the NO2 atom of NAD (Fig. 5).

C3stau2 Moves to Enclose the NAD—The structure of C3stau2 shows significant conformational change on binding NAD. The structure of C3bot1 in complex with NAD, when aligned along helices 2–4, shows a movement of a large domain comprising β2, α5, β7, and β8 toward the NAD binding cleft, closing the protein structure around the NAD. Fig. 5 shows that C3stau2 also undergoes a similar clamping movement involving the variable domain (helices α5 and α6 along with strands β7 and β8) and helix α3 to enclose its substrate. The variable domain can be seen to rotate up to 4° about the catalytic residue, Glu-180, toward the binding site. As with C3bot1, this change distorts the C-terminal end of strand β1 toward the NAD, in this case bringing Arg-85 and Leu-86 into range for NAD binding. Also, helices α3 and α4 on the other side of the NAD binding cleft can be seen rotating a similar amount in the opposite direction to close the gap further.

Fig. 4 shows that these large structural changes bring the α carbons of 7 of the 10 NAD binding residues between 0.82 and 1.51 Å closer to the NAD. This in turn allows the critical side chains to be brought within bonding distance of the NAD. Most notable are residues Arg-85 and Arg-48, which have been shown by mutational analysis to be essential for NAD binding in C3stau2 (11). A change in the side-chain conformation and a main chain Cα shift of ~0.82 Å in Arg-85 on strand β1 moves its NH2 atom 2.95 Å, allowing interaction with the adenosine phosphate AO1 atom of NAD. For Arg-48 on helix α3, a change in side chain conformation and a Cα shift of 1.51 Å moves its NH1 atom 2.16 Å, allowing bonding with the NAD AO2 atom.

Analysis of the Common NAD Binding Site of the C3-like and Binary Toxins—The structures of the C3-like and binary toxins determined so far reveal similar, but not identical NAD binding sites. Table III shows the residues of C3stau2, C3bot1 (23), and the binary toxins, Iota toxin from C. perfringens (25, 38) and VIP2 from B. cereus (24), that are involved in binding NAD/NDH. Superposition of the binding sites from all four structures shows that the nicotinamide portion of the NAD is in a similar orientation, while the central phosphates and the adenine differ slightly in their positions. The NAD binding site is characterized not only by the ARTT and PN loops but also by two arginines, conserved in the C3-like and binary toxins, and a third, conserved in the C3-like toxins.

The most conserved feature of the binding site is the positioning of the nicotinamide moiety by the main chain nitrogen and oxygen atoms of the residue after the conserved arginine equivalent to Arg-85 in C3stau2. Because the identity of this residue varies from glycine in C3bot1 to tryptophan in VIP2, it is likely that any residue that can adopt the appropriate backbone conformation will support this interaction. Apart from this residue and the catalytic glutamate, the only other conserved interaction is some stabilization/orientation of the adenine ring, which is positioned by hydrogen bonds in C3stau2, C3bot1, and the Iota toxin and by hydrophobic packing in C3bot1 and VIP2.

In C3stau2, three arginines conserved among the C3-like toxins are seen to interact with the central phosphates of the NAD (Fig. 6). These interactions have been proposed to be involved in increasing affinity for the substrate (24, 38). Mutational analyses of these residues have been reported for C3stau2 (11) and C3bot1 (23) as well as for the Iota toxin (25, 38) and confirm the importance of these arginines in NAD binding or hydrolysis. Surprisingly, comparison of the structures of all four enzymes shows that the interactions between these arginines and NAD are not always identical (Fig. 6 and Table III). The two arginines conserved in the C3-like and binary toxins interact with the central phosphates in all the structures except VIP2. VIP2 does undergo a similar conformational change to C3stau2 upon NAD binding, and although it retains these arginines in positions similar to those of C3stau2,
they are not close enough to provide the same type of interactions. This difference seems to correlate with the difference in the orientation of the NAD in the binding pocket of VIP2 and does not necessarily reflect the nature of transition state binding, which could be similar for all four enzymes. Alternatively, the first of the serine in the STX motif for Iota toxin NAD hydrolysis (37), which is not the case in C3stau2 and C3bot1 (11, 23), may indicate a difference in the mode of transition state stabilization between the C3-like and binary toxins.

The arginine at position 3 is only conserved in the C3-like toxins but also occurs in the binary toxins VIP2 and C2 from C. botulinum and may be presumed to have a conserved function. This arginine has also been shown to be essential for NAD binding to C3stau2 (11) and is seen binding to the AO2 atom of NAD. However, in C3bot1, despite having the potential to bind to the central phosphate, it adopts a different conformation, and its C8 and N atoms engage in van der Waals interactions with the AN6 and AC6 atoms of the NAD. In VIP2, the guanidinium group makes π–π interactions with the adenine ring, suggesting that this residue has several possible means of positioning the NAD or that these orientations are preliminary to further change on hydrolysis. The variation in the orientation of this residue also coincides with the larger divergence of this (adenine) end of the binding pocket.

Interactions of C3stau2 with the RhoA and RalA—Although structural data and mutational analysis have gone a long way toward elucidating the mechanism of NAD cleavage and ADP-ribosylation among the C3-like toxins, only Tyr-175 in C3stau2 (11) and its equivalent, Phe-209, in C3bot1 (39) have been identified as essential for binding RhoA by mutational analysis. The variation of this residue between the two enzymes may have some part to play in protein target specificity. However, more detailed mutational analysis of the C. difficile Iota toxin suggests that helix α8 (equivalent to helix α3 in C3stau2) may also be partially responsible for the binding of protein substrate, which for the Iota toxin is actin (25). Also, two residues from this helix, Tyr-246 and Asn-255, in structurally equivalent positions to Ser-40 and Asn-44 in C3stau2 have been identified as residues important for protein substrate binding (38). Asn-255 was additionally identified as being important for protein substrate specificity (38). Interestingly, VIP2 and Iota toxin, which bind actin as a substrate, align very well along helix 3, which share actin as a substrate, align very well along helix 3, substrate specificity (38). Interestingly, VIP2 and Iota toxin, which for the Iota toxin is actin (25). Also, two residues from this helix, Tyr-246 and Asn-255, in structurally equivalent positions to Ser-40 and Asn-44 in C3stau2 have been identified as being important for protein substrate binding (38). Asn-255 was additionally identified as being important for protein substrate specificity (38). Interestingly, VIP2 and Iota toxin, which share actin as a substrate, align very well along helix 3, which share actin as a substrate, align very well along helix 3, substrate specificity (38). Interestingly, VIP2 and Iota toxin, which for the Iota toxin is actin (25).

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The Crystal Structure of C3stau2 from *Staphylococcus aureus* and Its Complex with NAD

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