# Structure−Function Analysis of the Rho-ADP-Ribosylating Exoenzyme C3stau2 from *Staphylococcus aureus*

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ABSTRACT: Exoenzyme C3stau2 from *Staphylococcus aureus* is a new member of the family of C3-like ADP-ribosyltransferases that ADP-ribosylates RhoA, -B, and -C. Additionally, it modifies RhoE and Rnd3. Here we report on studies of the structure−function relationship of recombinant C3stau2 by site-directed mutagenesis. Exchange of $Glu^{180}$ with leucine caused a complete loss of both ADP-ribosyltransferase and NAD glycohydrolase activity. By contrast, exchange of the glutamine residue two positions upstream ($Gln^{178}$) with lysine blocked ADP-ribosyltransferase activity without major changes in NAD glycohydrolase activity. NAD and substrate binding of this mutant protein was comparable to that of the recombinant wild type. Exchange of amino acid $Tyr^{175}$, which is part of the recently described "ADP-ribosylating toxin turn−turn" (ARTT) motif [Han, S., Arvai, A. S., Clancy, S. B., and Tainer, J. A. (2001) *J. Mol. Biol. 305*, 95−107], with alanine, lysine, or threonine caused a loss of or a decrease in ADP-ribosyltransferase activity but an increase in NAD glycohydrolase activity. Recombinant C3stau2 Tyr175Ala and Tyr175Lys were not precipitated by matrix-bound Rho, supporting a role of $Tyr^{175}$ in protein substrate recognition. Exchange of $Arg^{48}$ and/or $Arg^{85}$ resulted in a 100-fold reduced transferase activity, while the recombinant C3stau2 double mutant R48K/R85K was totally inactive. The data indicate that amino acid residues $Arg^{48}$, $Arg^{85}$, $Tyr^{175}$, $Gln^{178}$, and $Glu^{180}$ are essential for ADP-ribosyltransferase activity of recombinant C3stau2 and support the role of the ARTT motif in substrate recognition of RhoA by C3-like ADP-ribosyltransferases.

Various bacterial protein toxins and/or exoenzymes interfere with eukaryotic cell functions by modifying essential eukaryotic regulatory proteins. An example of this type of toxin action is the ADP ribosylation of Rho-GTPases by C3-like transferases. *Clostridium botulinum* exoenzyme C3 (C3bot) is the prototype of a family of Rho-ADP-ribosylating transferases encompassing additional exoenzymes from *Clostridium limosum*, *Bacillus cereus*, and *Staphylococcus aureus* (*1*−*8*). All these exoenzymes have molecular masses of 23−28 kDa and are single-chain basic proteins, and their level of amino acid sequence identity is in the range of 35−65%. Alignments show that short peptide stretches or at least key amino acids are conserved in all of them. Recently, the crystal structure of C3bot was determined (*9*), allowing more precise insights into the structural and functional relationships among C3-like ADP-ribosyltransferases. The active site of C3bot is formed by an α-helix bent over a *β*-strand, surrounded by two *β*-strands in which key amino acid residues are located that are conserved in most ADP-ribosyltransferases. Moreover, a new motif in the structure of the C3bot exoenzyme was described and termed the "ARTT" (ADP-ribosylating toxin turn−turn) motif. This motif of two short amino acid stretches, covering residues 167−170 and 171−174 of C3bot, was suggested to be responsible for substrate recognition and important for the catalytic activity of the C3bot exoenzyme (*9*). Further insights into the structure−function relationship of C3bot were obtained by the analysis of the crystal structure of the vegetative insecticidal protein (VIP2) from *B. cereus* (*10*). VIP is a binary toxin and consists of the membrane-binding component VIP1 and the actin−ADP-ribosylating component VIP2. VIP2 is approximately 30% identical with exoenzyme C3 from *C. botulinum* on the amino acid sequence level and possesses several key residues in common with C3bot. Importantly, VIP2 has been crystallized in a complex with NAD, allowing the identification of NAD-interacting residues of VIP2 (*10*). Recently, we identified a novel C3-like ADP-ribosyltransferase from *S. aureus* (*7*). The amino acid sequence of this exoenzyme is ∼78% identical with that of EDIN from *S. aureus* and was called C3stau2 (accordingly, EDIN was called C3stau1). C3stau2 differs from other C3-like transferases in its substrate specificity. It modifies RhoA, -B, and -C and additionally RhoE and Rnd3 (*7*), which are constitutively active variants of Rho GTPases and were suggested to act as antagonists of Rho-family proteins (*11*). Here we report on the structure−function analysis of the recombinant C3stau2 exoenzyme (rC3stau2)[1] that is based on site-directed mutagenesis.

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## MATERIALS AND METHODS

*Materials and Chemicals.* Oligonucleotides were obtained from MWG (Ebersberg, Germany). The pGEX-2T vector and the glutathione *S*-transferase Gene Fusion system were from Pharmacia Biotech (Uppsala, Sweden). The Quick-Change Kit was from Stratagene (Heidelberg, Germany). Restriction endonucleases were from NEB (Schwalbach, Germany), and [$^{32}$P]NAD was from NEN. All other chemicals were from commercial sources.

*Construction of Mutant rC3stau2.* rC3stau2 mutant proteins were constructed by site-directed mutagenesis with the pGEX-2T-C3stau2 plasmid (*7*) as a template and the respective oligonucleotides using the Quick-Change Kit according to the manufacturer's instructions. From the two complementary primers that were needed, only one is listed: W15A, 5′-GTT GAA GCT ACT AAA GCA GGA AAC TCA TTA ATA AAG-3′; W15F, 5′-GTT GAA GCT ACT AAA TTT GGA AAC TCA TTA ATA AAG-3′; R48K, 5′-TAC TCC TCT AAA ATC AGC AAA TGG TG-3′; R85K, 5′-CTA TGT ATA CAA ATT ATT AAA TTT AGA C-3′; S137A, 5′-GAA AAT GGC TAC GCA AGT ACA CAA CTA G-3′; S138A, 5′-TGG CTA CTC TGC AAC ACA ACT AGT TAG-3′; T139V, 5′-CTA CTC TAG TGT TCA ACT AGT TAG TGG-3′; Q140A, 5′-CTC TAG TAC AGC ACT AGT TAG TGG TGC-3′; Y175T, 5′-GAG TTA ACA GCA ACT CCA GGT CAA CAA G-3′; Y175A, 5′-GTT AAC AGC AGC ACC AGG TCA ACA AG-3′; Y175K, 5′-GTT AAC AGC AAA ACC AGG TCA ACA AG-3′; Q178K, 5′-CAG CAT ACC CAG GTA AAC AAG AAG TTC TTT TG-3′; and E180L, 5′-CCA GGT CAA CAA TTA GTT CTT TTG CCT AG-3′. The mutated plasmids were transformed in *Escherichia coli* XL-1 blue supercompetent cells (Stratagene), and all mutations were confirmed by DNA sequencing (Cycle Sequencing Ready Reaction Kit, Perkin-Elmer).

*Expression and Purification of Recombinant C3stau2.* The C3stau2 wild type or various C3stau2 mutant proteins were expressed as recombinant glutathione *S*-transferase fusion proteins in *E. coli* as described previously (*7*). For cleaving the GST carrier, the recombinant GST fusion proteins were incubated with thrombin (3.25 NIH units/mL) in a buffer containing 150 mM NaCl, 2 mM $CaCl_2$, and 5 mM TEA (pH 7.4) for 45 min at 37 °C. Thrombin was removed by incubation with benzamidine beads (Amersham Pharmacia Biotech), which were precipitated by centrifugation. GST- and thrombin-cleaved rC3stau2 proteins were subjected to SDS−PAGE to control the purification and cleavage procedure.

*ADP Ribosylation Reaction.* RhoA (2 $\mu$M) was incubated with the rC3stau2 wild-type or mutant protein at various concentrations in a buffer containing 50 mM HEPES (pH 7.3), 2 mM $MgCl_2$, 20 $\mu$M [*adenylate*-$^{32}$P]NAD, and 100 $\mu$g/mL BSA at 37 °C for up to 4 min. Radiolabeled proteins were resolved by SDS−PAGE and subsequently analyzed by phosphorimaging. The amount of incorporated [*adenylate*-$^{32}$P]ADP−ribose moiety was calculated from PhosphorImager data.

*SDS−PAGE.* SDS−polyacrylamide gel electrophoresis was performed according to the standard method of Laemmli. Gels were stained with Coomassie Brilliant Blue R250, dried, and further analyzed with the PhosphorImager SI from Molecular Dynamics.

*NAD Glycohydrolase Reaction.* For detection of glycohydrolase activity of the recombinant proteins, various concentrations of the exoenzyme were incubated with 200 $\mu$M [*adenylate*-$^{32}$P]NAD in a buffer containing 50 mM HEPES (pH 7.3) and 2 mM $MgCl_2$ at 37 °C for 1 h. Aliquots (5 $\mu$L) of the reaction mix were separated by TLC on Silica Gel $60_{F254}$ (Merck) with 66% 2-propanol and 0.33% ammonium sulfate, followed by phosphorimaging. The amount of cleaved [*adenylate*-$^{32}$P]ADP−ribose moiety was again calculated from PhosphorImager data.

*Tryptophan Quenching Assay.* Recombinant wild-type or mutant C3stau2 (12 $\mu$M each) was prewarmed to 25 °C in 50 mM HEPES (pH 7.3). After NAD (5 $\mu$M to 1 mM) had been added, the solution was mixed and the tryptophan fluorescence was measured by UV spectroscopy in an LS-50B spectrometer (Perkin-Elmer) at an excitation wavelength of 278 nm and an emission wavelength of 304 nm. From these data, values of a tryptophan solution of the same concentration were subtracted. The resulting data were denoted as "quench". The $K_D$ for NAD for the rC3stau2 wild-type protein was calculated from the data according to the Lineweaver−Burk method.

*Precipitation Assay.* Wild-type RhoA was expressed as a recombinant glutathione *S*-transferase fusion protein in *E. coli* and coupled to glutathione beads. Two micrograms of RhoA was incubated with 300 ng of the rC3stau2 wild-type or mutant protein as indicated in a buffer containing 50 mM HEPES (pH 7.3), 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, and Nonidet P40 (final concentration of 0.5%) for 45 min at 4 °C. Beads were washed three times with buffer and subjected to SDS−PAGE. After proteins had been transferred onto nitrocellulose, the bound exoenzyme was detected with a specific polyclonal antibody (rabbit) against the rC3stau2 wild-type protein.

## RESULTS

*Alignment of C3stau2 with Other C3-like ADP-Ribosyltransferases.* Comparison of C3stau2 with related C3-like ADP-ribosylating toxins reveals several conserved residues (Figure 1). A conserved glutamate ($Glu^{180}$), termed the "catalytic glutamic acid", is localized at the C-terminus and is flanked by a conserved glutamine ($Gln^{178}$) two positions upstream. The former residue is essential for the enzymatic activity of all known ADP-ribosyltransferases, while the latter one is present in all ADP-ribosyltransferases that modify their target proteins at an asparagine residue. The "STS" motif, known to be critical for both the enzymatic activity and structural integrity of several ADP-ribosylating toxins, is changed to an "STQ" motif ($Ser^{138}$-$Thr^{139}$-$Gln^{140}$) in C3stau2 (Figure 1) (*12*). Moreover, $Arg^{45}$ and $Arg^{85}$ of C3stau2 are conserved between C3-like ADP-ribosyltransferases. At the N-terminus, a conserved tryptophan ($Trp^{15}$) is present. To investigate the role of these residues in Rho and NAD binding and in enzymatic activity, several rC3stau2 mutant proteins were expressed and purified as GST fusion proteins from *E. coli* (Figure 2).

*Characterization of the Recombinant C3stau2 Wild Type and Mutants.* First, we tested the ADP-ribosyltransferase activity of rC3stau2 WT. Therefore, no differences between

[1] Abbreviations: rC3stau2, recombinant C3stau2 that was purified from *E. coli*; rC3stau2 WT, recombinant C3stau2 wild-type toxin; rGST−C3stau2 WT, recombinant C3stau2 wild-type toxin, coupled to glutathione *S*-transferase.

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                          15                  48
C3stau2          12..ATKWGNS....43..INTPLRSANG...52
EDIN (C3stau1)   12..ATKWGNK....43..INGPLRLAGG...52
C3bot            15..AKAWGNA....46..INGPLRANQG...55
C3lim            15..ARAWGDK....46..INGPLRANQG...55
C2I             226..SEAWGAE...260..INSYLRNNRV..269
VIP2            276..AHSWGMK...310..INNYLRNQGG..319
Iota a          220..GDLWGKE...254..INNYLISNGP..263
CDTa            267..GDSWGKA...301..INNYLISNGP..310

                      85          137 138 139 140        175 178 180
C3stau2          83..VYRLL...136..YSSTQLV...173..TAYPGQQEVL..182
EDIN (C3stau1)   83..VYRLL...136..YSSTQLV...173..TAYYGQQEVL..182
C3bot            86..LFRGD...132..YISTSLM...167..SAFAGQLEML..176
C3lim            86..LFRGD...132..YISTSLM...167..STFKGQLEVL..176
C3cer                                       ..TAYPGQYELL..
C2I             297..AYRRV...346..FSSTSLK...382..SGFQDEQEIL..391
VIP2            347..VYRWC...384..YMSTSLS...421..GGFASEKEIL..430
Iota a          293..VYRRS...336..FISTSIG...373..PGYAGEYEVL..382
CDTa            343..VYRRS...386..FISTSIG...423..PGYAGEYEVL..432
                                                 T1  T2
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FIGURE 1: Alignment of the partial amino acid sequence of ADP-ribosylating toxins. Conserved residues are marked in gray, and numbers indicate their positions in the whole protein. The numbers above the C3stau2 sequence indicate the positions of residues that have been exchanged (note that the counting of amino acids in C3bot is without the signal sequence of 40 residues). Accession numbers are as follows: AJ277173 for C3stau2 (*S. aureus* strain HMI6), P24121 for EDIN (epidermal cell differentiation inhibitor, C3stau1) (*S. aureus* strain E1), P15879 for C3bot (*C. botulinum*), X87215 for C3lim (*C. limosum*), AAB 33136.1 for C3cer (partial peptide sequence, *B. cereus* strain 2339), AJ224480 for C2I (enzymatic fragment C2I from *C. botulinum*), X73562 for Iota a (enzymatic fragment iota A from *Clostridium perfringens*), and O32738 for CDTa (ADP-ribosyltransferase from *Clostridium difficile*). For VIP2 (*B. cereus*), no accession number is available. Turn 1 (T1) and turn 2 (T2) of the ARTT motif are denoted.

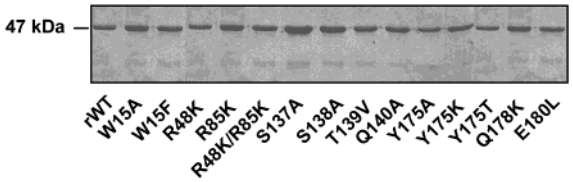


FIGURE 2: Expression and purification of rC3stau2 wild-type and mutant proteins. Proteins were expressed as GST fusion proteins with a molecular mass of 49 kDa. Protein (1.5 $\mu$g) was loaded onto the gel, and the Coomassie-stained gel is shown.

the activity of rGST−C3stau2 and thrombin-cleaved rC3stau2 were detected (data not shown). The specific ADP-ribosyltransferase activity was 159 mol of ADP-ribosylated RhoA (mol of toxin)$^{-1}$ min$^{-1}$ (Figure 3).

Then, we studied the role of C-terminally located conserved residues Tyr$^{175}$, Gln$^{178}$, and Glu$^{180}$. The equivalent residues of C3bot (e.g., Phe$^{169}$, Gln$^{172}$, and Glu$^{174}$) are essential parts of the bipartite "ADP-ribosylating toxin turn−turn" (ARTT) motif (*9*). Tyr$^{175}$ of C3stau2 was changed to alanine, lysine, and threonine. Whereas the ADP-ribosyltransferase activity of rC3stau2 Y175T was 60-fold lower than that of the wild type, the Y175A and Y175K mutants were more than 10 000 times less active. Exchange of Gln$^{178}$ (Q178K) and Glu$^{180}$ (E180L) of C3stau2 caused a similar decrease in activity (Figure 3 and Table 1). The data suggest that these three residues are absolutely essential for the ADP ribosylation of RhoA. Next, we studied the role of residues 138−140 in enzyme activity. The exchange of Gln$^{140}$ or Ser$^{138}$ with alanine or Thr$^{139}$ with valine resulted in only a slight reduction in the transferase activity, indicating a minor role of these residues in the catalysis of the ADP ribosylation reaction (Table 1). By contrast, Arg$^{48}$ and Arg$^{85}$ are crucial for enzyme activity. Both residues were individually changed

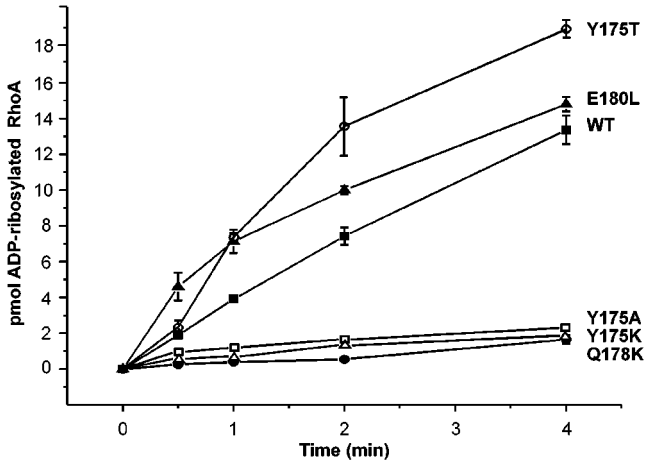


FIGURE 3: Time-dependent ADP ribosylation of recombinant RhoA. RhoA (2 $\mu$M) was incubated with 1 nM rC3stau2 WT (WT) or rC3stau2 Y175T (50 nM), Y175A (500 nM), Y175K (500 nM), Q178K (10 $\mu$M), or E180L (10 $\mu$M) in the presence of [$^{32}$P]NAD for up to 4 min. Radiolabeled probes were subjected to SDS−PAGE, and the amount of RhoA that was modified was quantified by phosphorimaging.

to lysine. The respective mutants exhibited a 100-fold reduced ADP-ribosyltransferase activity, while the double mutant was totally inactive (Table 1). Finally, we were interested in the role of Trp$^{15}$ in C3stau2. The equivalent tryptophan residue in VIP2 from *B. cereus* appears to be involved in the correct positioning of the nicotinamide moiety during the cleavage of NAD and the transfer of the ADP−ribose moiety onto the acceptor amino acid (*10*). Therefore, we changed Trp$^{15}$ to phenylalanine or alanine. Both mutations resulted in a slight reduction in the ADP-ribosyltransferase activity (Table 1).

Table 1: Properties of the Recombinant C3stau2 Wild Type (rWT) and Mutant Recombinant C3stau2[a]

| toxin | ADP-ribosyltransferase activity (relative activity in %) | NAD glycohydrolase activity (relative activity in %) | level of NAD quenching | level of RhoA binding |
|---|---|---|---|---|
| rWT | 100 | 100 | ++ | + |
| W15F | 74 | 84 ± 1.7 | nd | nd |
| W15A | 22 | 23 ± 0.2 | nd | nd |
| R48K | 1.6 | 4.4 ± 0.3 | − | nd |
| R85K | 1.5 | 5.6 ± 0.5 | − | nd |
| R48K/R85K | <0.01 | 1.5 ± 0.15 | − | nd |
| S137A | 97 | 85 ± 1.3 | nd | nd |
| S138A | 20 | 54 ± 8.2 | nd | nd |
| T139V | 13 | 57 ± 3.3 | nd | nd |
| Q140A | 57 | 49 ± 0.2 | nd | nd |
| Y175T | 1.7 | 606 ± 1.0 | ++ | + |
| Y175A | <0.01 | 170 ± 1.0 | nd | − |
| Y175K | <0.01 | 152 ± 1.0 | nd | − |
| Q178K | <0.01 | 40 ± 0.3 | + | + |
| E180L | <0.01 | 1.6 ± 0.17 | − | + |

[a] The specific ADP-ribosyltransferase activity of rC3stau2 WT was 159 mol of ADP-ribosylated RhoA (mol of toxin)$^{-1}$ min$^{-1}$. Transferase activities were measured in triplicate by exoenzyme-catalyzed labeling of recombinant RhoA in the presence of [*adenylate*-$^{32}$P]NAD. The concentrations of the exoenzyme were as follows: 1 nM rC3stau2 WT, 5 nM W15A, 1 nM W15F, 50 nM Y175T, 50 nM R48K, 50 nM R85K, 10 $\mu$M R48K/R85K, 2 nM S137A, 5 nM S138A, 5 nM T139V, 5 nM Q140A, 500 nM Y175A and Y175K, and 10 $\mu$M Q178K and E180L. Data were calculated from the regression curves of the time-dependent ADP ribosylation of recombinant RhoA; therefore, no standard deviation is given for the transferase activity. Relative enzyme activity was calculated with the activity of the rC3stau2 wild type taken to be 100%. The specific NAD glycohydrolase activity of rC3stau2 WT was 0.61 ± 0.02 mol of hydrolyzed NAD (mol of toxin)$^{-1}$ min$^{-1}$. For determination of the NAD glycohydrolase activities, 200 $\mu$M [*adenylate*-$^{32}$P]NAD were incubated with rC3stau2 WT, rC3stau2 Y175A or Y175T (0.2 $\mu$M), rC3stau2 S137A, S138A, T139V, Q140A, Y175K, or Q178K (1 $\mu$M), or rC3stau2 R48K, R85K, R48K/R85K, or E180L (10 $\mu$M). Probes were taken and analyzed by TLC as described in Materials and Methods. Enzyme activity is given as the mean ± standard error from three independent experiments. For calculation of the relative enzyme activity, the specific activity of the rC3stau2 wild type was taken to be 100%. NAD quenching data are from Figure 4 (++, ~30% quenching; +, ~15% quenching; −, <5% quenching; nd, not determined). RhoA binding data are from Figure 5 (+, binding comparable to that of the wild type; −, no binding; nd, not determined).

*NAD Glycohydrolase Activity.* All ADP-ribosyltransferases are able to catalyze the hydrolysis of NAD in the absence of a specific protein substrate. Therefore, we tested rC3stau2 WT and the mutant proteins described above for their NAD glycohydrolase activity. Although rC3stau2 Q178K had no detectable ADP-ribosyltransferase activity, it still possessed NAD glycohydrolase activity, which was reduced by ~50% (Table 1). Mutants that were defective in ADP-ribosyltransferase activity, e.g., R48K, R85K, R48K/R85K, and E180L, exhibited considerably decreased NAD glycohydrolase activities (Table 1). Surprisingly, there was one exception. The exchange of Tyr$^{175}$ with threonine, alanine, or lysine resulted in an increase in the specific NAD glycohydrolase activity. The NAD glycohydrolase activity of the other rC3stau2 mutants that were tested was only weakly affected (Table 1).

*Tryptophan Quenching of rC3stau2.* It is described for the C3-like transferase from *C. limosum* (*13*) that tryptophan fluorescence quenching is useful for analysis of NAD binding and determination of NAD affinity. Trp$^{15}$ is the only tryptophan residue in C3stau2 and is suitable for studies on NAD binding. Addition of increasing concentrations of NAD

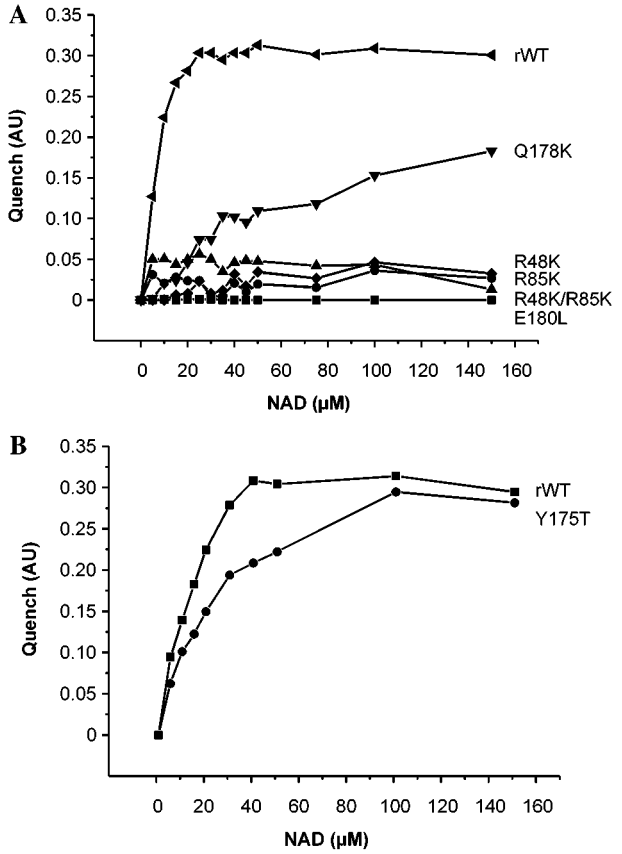


FIGURE 4: (A and B) Thrombin-cleaved rC3stau2 WT or ADP-ribosyltransferase defective mutants (12 $\mu$M each) were incubated with increasing concentrations of NAD (0−150 $\mu$M) in 50 mM HEPES (pH 7.3) at 25 °C, and the level of NAD-dependent quenching of tryptophan fluorescence was determined. The data were corrected using a tryptophan solution with the same concentration without exoenzyme as described. The level of quenching was given as arbitrary units (AU).

to rC3stau2 WT caused quenching of the tryptophan fluorescence (Figure 4A), indicating binding of NAD to the transferase. Addition of buffer instead of NAD resulted in stable tryptophan fluorescence during the whole experiment, indicating that the transferase is stable toward UV light (data not shown). Using the Lineweaver−Burk plot, the $K_D$ for NAD binding was calculated to be ~20 $\mu$M for rC3stau2 WT. Moreover, rC3stau2 Y175T and Q178K exhibited tryptophan quenching after addition of NAD (Figure 4A,B and Table 1). These data are in line with the NAD glycohydrolase activity of these mutants, supporting the view that the transferase activity was blocked but the ability to bind and to hydrolyze NAD is only weakly affected. By contrast, all other mutants defective in ADP-ribosyltransferase activity (e.g., rC3stau2 R48K, R85K, R48K/R85K, and E180L) exhibited no tryptophan quenching (Figure 4A and Table 1). Even in the presence of NAD at rather high concentrations (up to 1 mM), no quenching greater in magnitude than that caused by NAD itself was found (data not shown), indicating that these rC3stau2 mutants did not bind NAD.

*Binding of rC3stau2 Mutant Proteins to RhoA.* To examine the ability of enzymatically inactive rC3stau2 mutants to bind RhoA, we performed precipitation assays with these proteins. First, we tested the ability of the C3stau2 antibody to precipitate the rC3stau2 mutants tested in this assay from

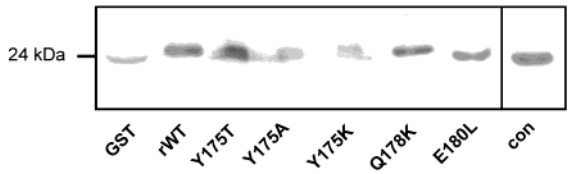


FIGURE 5: Precipitation assay of rC3stau2 Y175T, Y175A, Y175K, Q178K, E180L, or WT with immobilized recombinant RhoA. Glutathione *S*-transferase RhoA beads were incubated with the exoenzymes, washed, and subjected to SDS−PAGE. After being blotted onto nitrocellulose, the bound protein was detected by a specific antibody against rC3stau2. GST-loaded beads were used as a control for unspecific binding (GST), and rC3stau2 WT was applied as a control for the antibody (con).

solution. Thereby, no differences in the amount of precipitated recombinant toxin (wild type or mutant) could be detected (data not shown), indicating that the antibody recognizes all proteins equally well. Also, no differences in the detection of blotted proteins could be seen (data not shown). As shown in Figure 5, rC3stau2 Y175T, Q178K, and E180L were capable of binding to immobilized RhoA. The amount of bound protein was comparable to that of rC3stau2 WT, suggesting a similar binding affinity for their protein substrate RhoA. Interestingly, the level of binding of rC3stau2 Y175K and Y175A to RhoA was decreased and made comparable to the level of unspecific binding to GST beads alone.

## DISCUSSION

Recently, the crystal structure analysis of the Rho-ADP-ribosylating exoenzyme C3bot has been reported (*9*). Moreover, further insights into the structure−function relationship of C3-like exoenzymes were obtained by determining the crystal structure of the related ADP-ribosyltransferase VIP2 (*10*). Importantly, the actin−ADP-ribosylating VIP2, the amino acid sequence of which is ~30% identical with that of C3bot, was cocrystallized with NAD, allowing the positioning of residues which participate in binding and hydrolysis of NAD. On the basis of these crystal structure data, we performed a structure−function analysis of C3stau2 from *S. aureus* strain HMI6, a novel C3-like exoenzyme that was identified recently (*7*). As known for other ADP-ribosylating toxins, a C-terminally located glutamic acid residue ($Glu^{180}$ in C3stau2) plays a pivotal role in the catalytic mechanism of ADP-ribosyltransferase and NAD glycohydrolase activities. Exchange of the "catalytic" $Glu^{180}$ with leucine led to a nearly inactive rC3stau2. In the case of the related VIP2 toxin, the side chain of equivalent glutamic acid residue $Glu^{428}$ forms a hydrogen bond with the O2′ hydroxyl of the nicotinamide ribose, thereby increasing electron density on the ring and stabilizing the oxocarbenium ion that arises during the reaction (*10*). A similar mechanism is most likely functioning in C3bot (*9*) and C3stau2. Unlike the actin−ADP-ribosylating toxin VIP2, Rho-specific C3-like ADP-ribosyltransferases share a conserved glutamine residue instead of a glutamate that is located two residues upstream of the catalytic glutamic acid. In the VIP2 transferase, the side chain of $Glu^{426}$ is located outside the NAD binding cleft and may act in deprotonation of the side chain of actin $Arg^{177}$, which is the acceptor amino acid for the ADP−ribose moiety (*10*). In the case of C3bot, a comparable role in substrate recognition was postulated for the glutamine in position 172.[2] In C3bot, the side chain of $Gln^{172}$ is suggested to form hydrogen bonds between the carbonyl and the amide groups of Asn41 of RhoA. Mutation of the equivalent $Gln^{178}$ of rC3stau2 to lysine resulted in a complete loss of ADP-ribosyltransferase activity. Interestingly, this mutant did not show any difference in the binding to RhoA as determined in the pull-down assay. Thus, it seems that the side chain of $Gln^{178}$ is necessary for positioning of the correct ternary complex consisting of RhoA, NAD, and the exoenzyme. Furthermore, it seems that the side chain plays a more subordinate role in the recognition of and binding to RhoA. Han et al. (*10*) proposed from crystal structure analysis that C3bot $Gln^{172}$ is located outside the NAD-binding cleft. Two results in the present report support this notion. First, exchange of rC3stau2 $Gln^{178}$ with lysine did not block NAD glycohydrolase activity, and second, the tryptophan fluorescence of rC3stau2 Q178K was significantly quenched by addition of NAD, indicating binding of NAD.

Han et al. (*9*) suggested that C3bot and VIP2 are members of a subgroup of transferases possessing an ADP-ribosylating toxin turn−turn (ARTT) motif instead of an "active site loop" as observed in diphtheria toxin, *E. coli* heat labile toxin, *Pseudomonas aeruginosa* exotoxin A, or pertussis toxin (*14*−*18*). In the transferases possessing the ARTT motif, two adjacent, protruding turns are localized in the enzyme core and are involved in substrate recognition and enzyme activity. For C3-like exoenzymes, it was proposed that an aromatic amino acid residue (C3bot $Phe^{169}$ and C3stau2 $Tyr^{175}$) in turn 1 is responsible for the recognition of RhoA through hydrophobic interactions between hydrophobic patches around acceptor amino acid $Asn^{41}$ (*9*, *19*). Turn 2 harbors a glutamine residue (in the case of C3-like ADP-ribosyltransferases) or a glutamate residue (in the case of actin-modifying ADP-ribosyltransferases) and the catalytic glutamate residue already described above. Here we show that the exchange of turn 1 residue $Tyr^{175}$ of rC3stau2 with lysine or alanine decreased the ADP-ribosyltransferase activity by ~10000-fold. In line with the notion that $Tyr^{175}$ is involved in protein substrate recognition, precipitation of rC3stau2 by immobilized RhoA was blocked after $Tyr^{175}$ was changed to alanine or lysine. By contrast, the NAD glycohydrolase activity was not reduced but rather increased by the amino acid exchanges. Exchange of $Tyr^{175}$ with threonine reduced ADP-ribosyltransferase activity by ~60-fold. In this case, Rho binding determined by the pull-down assay was still possible. Notably, the specific NAD glycohydrolase activity of rC3stau2 Y175T was 6-fold higher than that of the wild type. The tryptophan quenching assay revealed a slight decrease in the affinity of rC3stau2 Y175T for NAD. At present, we can only speculate that the reduction in NAD affinity and the increase in the rate of NAD turnover are involved in enhancement of the NAD glycohydrolase activity of rC3stau2 Y175T.

Many ADP-ribosyltransferases contain an STS motif upstream of the catalytic glutamate residue. In VIP2, the STS motif ($Ser^{386}$-$Thr^{387}$-$Ser^{388}$) is located at the end of the $\beta$11 strands and suggested to be involved in stabilizing the NAD-binding pocket (*10*). Moreover, $Ser^{386}$ forms a hydrogen bond with the catalytic glutamate. In C3bot, the STS motif is at

[2] Note that the numbering of amino acids for C3bot in this article is without the signal sequence of 40 residues.

the end of $\beta$3 and interacts via hydrogen bonds with Glu$^{174}$. It has been suggested that the C3bot Ser$^{136}$ residue is involved in the positioning of the catalytic glutamic acid for catalysis (*9*). In C3stau2, this motif is changed to an STQ motif (Ser$^{138}$-Thr$^{139}$-Gln$^{140}$). Moreover, it is preceded by another serine (Ser$^{137}$). Exchange of Ser$^{137}$, Ser$^{138}$, Thr$^{139}$, and Gln$^{140}$ in succession had no influence (Ser$^{137}$) or reduced ADP-ribosyltransferase activity only slightly. This suggests that the STS motif of rC3stau2 plays no important role in catalysis, as suggested for other ADP-ribosyltransferases (*20*).

The identification of Arg$^{48}$ and Arg$^{85}$ in C3stau2 as essential arginine residues is fully in agreement with the reported structures of C3bot and VIP2. Arg$^{48}$ is equivalent with Arg$^{51}$ and Arg$^{315}$ in C3bot and VIP2, respectively (*9*, *10*). In the structures of both enzymes, these arginine residues are part of a polar pocket in which the adenine ring of NAD fits. Accordingly, exchange of the residue in C3stau2 reduced ADP-ribosyltransferase activity and NAD glycohydrolase activity and prevented tryptophan quenching by NAD. All these effects are plausibly explained by a reduction in the NAD binding affinity of the mutant C3stau2. Arg$^{85}$ of C3stau2 is equivalent with Arg$^{88}$ and Arg$^{349}$ of C3bot and VIP2, respectively. In VIP2, Arg$^{349}$ seems to interact with the NAD phosphates. A similar role was proposed for Arg$^{88}$ in C3bot. As discussed for Arg$^{48}$, it is conceivable that the exchange of Arg$^{85}$ in C3stau2 leads to a decreased level of NAD binding of the exoenzyme and, therefore, to a decreased enzyme activity. Accordingly, double mutant rC3stau2, in which both residues (Arg$^{48}$ and Arg$^{85}$) were exchanged, exhibited a blockade in NAD binding and inhibition of NAD glycohydrolase and ADP-ribosyltransferase activities. These findings indicate that C3stau2 Arg$^{48}$ and Arg$^{85}$ may have equivalent functions like the respective arginine residues in the VIP toxin from *B. cereus*.

Taken together, the structure−function analysis by site-directed mutagenesis of rC3stau2 reported herein is largely in agreement with recent crystal structure analysis of the related exoenzymes C3bot and VIP2. Moreover, our kinetic data strongly suggest the involvement of the ARTT motif that was recently identified by crystal structure analysis in substrate recognition.

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