Characterization of the Catalytic Site of the ADP-Ribosyltransferase Closstridium botulinum C2 Toxin by Site-directed Mutagenesis

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The actin ADP-ribosylating Closstridium botulinum C2 toxin is a binary toxin composed of the binding component C2II and the enzyme component C2I. C2I ADP-ribosylates G-actin at arginine 177, resulting in the depolymerization of the actin cytoskeleton. Here, we studied the structure-function relationship of C2II by site-directed mutagenesis. Exchange of Glu388 to glutamine caused the complete loss of ADP-ribosyltransferase and NAD-glycohydrolase activities of C2I. In contrast, exchange of Glu387 to glutamine blocked ADP-ribosyltransferase but not NAD-glycohydrolase activity. Whereas photoaffinity labeling of the double mutant E387Q/E389Q C2I with [carbonyl-14C]NAD was blocked, labeling of the single C2I mutants was reduced (E389Q) or not changed (E387Q). Exchange of the STS motif (amino acid residues 348–350) of C2I caused a decrease in transferase activity by more than 99% (S350A), or did not affect activity (S350A). Exchange of Arg299 and Arg300 to lysine reduced transferase activity to <0.1 and ~35% of wild-type activity. The data indicate that the amino acid residues Glu388, Glu387, Ser348, and Arg299, which are conserved in various prokaryotic and eukaryotic arginine-modifying ADP-ribosyltransferases, are essential for ADP-ribosyltransferase activity of the enzyme component of C. botulinum C2 toxin.

Various bacterial exotoxins ADP-ribosylate eukaryotic proteins. These proteins are essential for signal transduction or cellular structure. With respect to their protein substrates, these toxins can be classified into at least four groups: (i) elongation factor 2 ADP-ribosylating toxins (e.g. diphtheria toxin) (1); (ii) heterotrimeric G-protein ADP-ribosylating toxins (e.g. cholera and pertussis toxin) (2); (iii) toxins (e.g. Closstridium botulinum C3 exoenzyme) that ADP-ribosylate small GTPases (3); and (iv) ADP-ribosyltransferases with actin as a substrate (4). The fourth group includes the clostridial ADP-ribosyltransferases C. botulinum C2 toxin (5), Closstridium perfringens iota toxin (6), Closstridium sprotiforme toxins (7), and an ADP-ribosyltransferase from Closstridium difficile (8).

The binary C. botulinum C2 toxin is composed of the binding component C2II (M₆₀ ~100,000) and the enzymatic component C2I (M₆₀ ~49,000). Both components are separate proteins and are neither covalently nor noncovalently linked (9). To elicit toxic effects, the trypsin-activated C2II (M₆₀ ~80,000) binds to the cell surface and forms a binding site for C2I (10). The toxin enters the cell via receptor-mediated endocytosis (11) followed by translocation of C2I into the cytosol. In the cell, the enzyme component ADP-ribosylates G-actin at arginine 177 (5, 12). Substrates of C2 toxin are βγ-non-muscle actin and γ-smooth muscle actin, but not α-actin isoforms. In contrast to C2 toxin, the related iota toxin ADP-ribosylates all actin isoforms (13). ADP-ribosylation inhibits actin polymerization and blocks the actin ATPase activity (5, 14). Moreover, ADP-ribosylation of actin blocks the nucleation activity of the gelsolin-actin complex (16). In intact cells, C2 toxin causes complete depolymerization of the actin cytoskeleton and eventually cell death (17).

Despite a rather poor amino acid sequence homology between the various bacterial ADP-ribosyltransferases, recent crystallographic data revealed a very similar tertiary structure between Pseudomonas aeruginosa exotoxin A (18), diphtheria toxin (19), Escherichia coli heat-labile enterotoxin (20), and pertussis toxin (21). The NAD-binding and catalytic site, which is formed by two antiparallel β-sheets flanked by two α-helices, appears to be highly conserved among all these ADP-ribosylating toxins. The crystallographic analyses corroborated earlier biochemical findings that indicated a highly conserved glutamic acid residue as the essential part of the transferase active site (22, 23). In addition, a second glutamic acid residue, located two residues upstream of the “catalytic” glutamate residue is conserved in many ADP-ribosyltransferases. It has been suggested that this glutamic acid residue is also important for the transferase reaction (24–26). Moreover, most toxins (with the exception of group (i) toxins), share a common arginine residue about 50–120 amino acids upstream of the glutamate residue and a highly conserved serine-threonine-serine (STS) motif between these two regions (26).

Recently, the sequence of the structural gene of the enzyme component of C. botulinum C2 toxin was reported (27). To compare functionally important domains of C2I with those of other transferases including iota toxin, we analyzed the structure-function relationship of the enzyme component of C2 toxin by site-directed mutagenesis. Here, we report that in addition to the conserved active site glutamic acid residue Glu388, a second glutamic acid residue Glu387 is essential for transferase activity but not for NAD-glycohydrolase activity of C2I. Moreover, the conserved STS motif and the arginine residue most
likely involved in NAD-binding of C2I are identified as residues Ser\(^{348}\)–Thr\(^{349}\)–Ser\(^{350}\) and Arg\(^{299}\), respectively.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides were obtained from BIG (Denzlingen, Germany). The pGEX-2T vector was included in the glutathione S-transferase Gene Fusion System from Pharmacia Biotech (Uppsala, Sweden), and the Quikchange Kit was from Stratagene (Heidelberg, Germany). Polymerase chain reactions were performed in the GeneAmp polymerase chain reaction system 2400 from Perkin-Elmer (Langen, Germany), and DNA sequencing was conducted with the Cycle Sequencing Ready Reaction Kit (ABI PRISM) from Perkin-Elmer. C.\(\text{botulinum}\) C2II was purified and activated with trypsin as described previously (10). The antiserum against C2I was raised in rabbits against the whole protein. Donkey anti-rabbit-antibody coupled to horseradish peroxidase and the enhanced chemiluminescence detection kit were from Amersham (Braunschweig, Germany). The nitrocellulose blotting-membrane was from Schleicher & Schuell (Dassel, Germany). Low molecular mass protein markers were obtained from Bio-Rad. Glutathione-Sepharose 4B was obtained from Pharmacia Biotech (Uppsala, Sweden).

**Active Site Mutations of C. botulinum C2 Toxin**

**C2**

295 LIAYRRVDG... 343 NLSFSSTSKT... 383 GFQDEQIEILNN

**Iota**

291 LIIVRSGSP... 333 YPHIITSITGVS... 374 GAYEYQVEVLN

**CT**

3 DKLYRADSR... 56 DDGYVTSISLR... 106 PHPDQQVSAAL

**C3**

84 ILFRGDPP... 129 EGYITSLMV... 168 AFAGQLEMLLP

**PT**

5 ATVYRYSDR... 47 NSAFVSTSSR... 123 LATYSEYLIAY

**DT**

17 FSSGHTKSP... 49 DWSGFYSTDNYK... 142 EGSYSEYVINN

**RT6.1**

142 HSVYRGTKT... 162 PGQFTSSSLKT... 203 FYPDQEYVLIP

**FIG. 1.** A, alignment of highly conserved amino acid residues (boldface) and common motifs of various ADP-ribosyltransferases. Numbers label the position of amino acid residues (C2, C.\(\text{botulinum}\) C2 toxin (GenBank\textsuperscript{TM} accession no. AJ224480); Iota, C.\(\text{perfringens}\) iota toxin (accession no. X73562); CT, cholera toxin (accession no. X09171); C3, C.\(\text{botulinum}\) C3 exoenzyme (accession no. X82715); PT, pertussis toxin (accession no. M13223); DT, diphtheria toxin (accession no. K01723); RT6.1, rodent T-cell RT6 antigen, all \(\alpha\) (accession no. X52082 (M31138)). B, alignment of amino acid sequences of C.\(\text{perfringens}\) iota a and C.\(\text{botulinum}\) C2I. Numbers label the position of amino acid residues. C.\(\text{botulinum}\) C2I (GenBank\textsuperscript{TM} accession no. AJ224480); C.\(\text{perfringens}\) iota a (accession no. X73562).

**FIG. 2.** Amino acid sequences of wild-type C2I and the respective mutants. Numbers indicate the positions of amino acids. Boldface letters mark the exchanged amino acid residues.

**FIG. 3.** Identification of mutant C2I proteins by SDS-PAGE. C2I proteins were expressed in \(E.\\text{coli}\) and purified as described. One \(\mu\)g of protein was loaded onto 11% SDS-PAGE and the proteins were stained with Coomassie Blue.

**Fig. 3**. Identification of mutant C2I proteins by SDS-PAGE. C2I proteins were expressed in \(E.\\text{coli}\) and purified as described. One \(\mu\)g of protein was loaded onto 11% SDS-PAGE and the proteins were stained with Coomassie Blue.
Active Site Mutations of C. botulinum C2 Toxin

psal, Sweden). Silica Gel 60 F254 TLC sheets were from Merck (Darmstadt, Germany). Cell culture medium was purchased from Biochrom (Berlin, Germany), fetal calf serum was obtained from PAN Systems (Aidenbach, Germany) and cell culture materials were purchased from Falcon (Heidelberg, Germany). Thrombin was obtained from Sigma (Deisenhofen, Germany).

Construction of C2I Mutants—The C2I mutants were constructed by site-directed mutagenesis with the pGEX2T-C2I plasmid (28) as a template and the respective oligonucleotides using the Quikchange kit according to the manufacturer’s instructions. The pGEX2T-C2I plasmid consists of the translated region of C2I (1296 base pairs) from C. botulinum strain 92-13 (GenBank accession no. AJ224480) as an insert in a pGEX-2T vector. For each mutant, two complementary synthetic oligonucleotides were needed (only one of the two complementary oligonucleotides is indicated). R299K, 5′-cta tta aca gta aag gta gat ggt att cc-3′; R300K, 5′-cta tta aca gta aag gta gat ggt att cc-3′; S347A, 5′-gaa aat tta tca ttc tct gt ct tct cta aac tcc ce-3′; T349V, 5′-gaa aat tta tca ttc tct gt ct tct cta aac tcc ce-3′; S350A, 5′-gaa aat tta tca ttc tct gt ct tct cta aac tcc ce-3′; S347T/350A, 5′-gaa aat tta tca ttc tct gt ct tct cta aac tcc ce-3′; S347T/350A, 5′-gaa ttt gaa cag gaa cag aag aat gca gta gat ggt att cc-3′; S347T/350A, 5′-gct gct aag aat gca gta gat ggt att cc-3′; E387Q, 5′-gga ttt gaa cag gaa cag aag aat gca gta gat ggt att cc-3′; E387Q, 5′-gga ttt gaa cag gaa cag aag aat gca gta gat ggt att cc-3′; E387Q, 5′-gga ttt gaa cag gaa cag aag aat gca gta gat ggt att cc-3′; E387Q, 5′-gga ttt gaa cag gaa cag aag aat gca gta gat ggt att cc-3′; E387Q, 5′-gga ttt gaa cag gaa cag aag aat gca gta gat ggt att cc-3′.

Expression and Purification of Recombinant C2I Proteins—C2I wild-type or the various C2I mutant proteins were expressed as recombinant glutathione S-transferase-fusion proteins in E. coli harboring the respective DNA fragment in the plasmid pGEX-2T. Bacteria were grown at 37 °C in LB medium containing 100 μg/ml ampicillin to an optical density (600 nm) of 0.8. isopropyl-1-thio-β-D-galactopyranosidase was added to a final concentration of 0.1 unit/ml and the cultures were incubated at 29 °C for another 20 h. Bacteria were sedimented at 7,700 × g (10 min, 4 °C) and resuspended in PBS, 1% Triton X-100. After sonication, the bacterial debris was sedimented at 12,000 × g (10 min, 4 °C). The supernatant was added to a 50% slurry of glutathione-Sepharose 4B in PBS (2 ml/100 ml), and incubated for 30 min at room temperature. After centrifugation at 500 × g (10 min, room temperature), the pellet was washed five times with 10 bed volumes of PBS and incubated with thrombin (3.25 NIH units/ml bead suspension) for cleavage of the fusion proteins from glutathione S-transferase. Thereafter, the supernatant was centrifuged at 500 × g (10 min, room temperature), and an aliquot of the resulting supernatant was subjected to a 12.5% SDS-PAGE followed by Western blot analysis with antiserum against C2I.

SDS-PAGE—SDS-PAGE was performed according to the methods of Laemmli (29). The gels were stained with Coomassie Brilliant Blue R-250.

ADP-ribosylation Assay—Recombinant C2I (at indicated concentrations) was incubated with human platelet cytosol (about 50 μg of protein) in 25 μl of buffer containing 35 mM HEPES (pH 7.5), 0.2 mM MgCl2, 0.4 mM dithiothreitol, and 0.5 mM NAD (about 25 nCi) for up to 10 min at 37 °C. Radiolabeled proteins were detected by 11% SDS-PAGE with subsequent phosphorimaging. The femtomole amount of incorporated ADP-ribose was calculated.

NAD-Glycohydrolase Assay—For detection of glycohydrolase activity the recombinant proteins (100 μg/ml) were incubated with 10 or 50 μM [3H]-ND (255 μCi NAD in 50 mM HEPES (pH 7.5) for 8 h. Five-μl aliquots of the reaction mixture (0.5 μg of protein) were separated by TLC on TLC aluminum sheets (Silica Gel 60 F254) with 66% 2-propanol, 0.33% acetic acid, and 0.1% ammonium sulfate and analyzed by phosphorimaging.

Cell Culture and Cytotoxicity Assay—CHO-K1 cells were cultivated in tissue culture flasks at 37 °C and 5% CO2 in Dulbecco’s minimal essential medium containing 5% heat-inactivated fetal calf serum, 2 mM l-glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were routinely trypsinized and reseeded twice a week. For cytotoxicity assays, cells were grown as subconfluent monolayers (about 106 cells/cm2) in 24-well plates containing coverslips and treated with 200 ng/ml activated C2II and 100 ng/ml of the respective C2I. Cells were washed with PBS, fixed in 4% paraformaldehyde in PBS for 30 min and washed, and the coverslips were embedded in Kaiser’s gelatin on glass slides.

Photoaffinity Labeling of C2I—Photoaffinity labeling was performed essentially as described previously (23). 2.5 μg of C2I were incubated in 50 μl of 25 mM ammonium bicarbonate (pH 7.8) with 50 μM [carbonyl-14C]NAD for 1 h at 4 °C. UV irradiation (254 nm, 3 cm distance, 3000 microwatts/cm2) was performed at 4 °C on a 96-well cell culture plate for 3 h. Twenty-μl aliquots were removed, and the proteins were separated on SDS-PAGE. Radioactive labeling was detected by phosphorimaging.

RESULTS

Characterization of the C2I Mutants—Sequence comparison of C2I toxins with iota toxin suggested that the catalytic site of C2I is located at its C terminus. From the sequence alignment of C2I, which we have recently cloned from C. botulinum strain 92-13, and Iota a, we proposed that Glu387 and Glu389, the “STS” motif (Ser348-Thr349-Ser350), and Arg299 are functionally important amino acid residues of C2I (Fig. 1). To test the hypothesis that these residues are essential for the catalysis of the ADP-ribosylation reaction and/or the NAD-binding of C2I, a series of mutations of C2I was performed (Fig. 2). These mutant proteins were expressed in E. coli and purified by using the glutathione S-transferase fusion protein system. After cleavage with thrombin, the proteins were analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 3, all mutant C2I proteins exhibited the expected molecular mass of ∼49 kDa. The band that runs just above the major band in each of the preparations was also present in various proteins, that were expressed and purified with the glutathione S-transferase gene fusion system in our laboratories. Next, the ADP-ribosyltransferase activities of the mutant C2I proteins were tested (Figs. 4 and 5 and Table I). Whereas wild-type C2I caused the [32P]ADP-ribosylation of β-γ-actin in human platelet cytosol, no labeling of actin was observed with E387Q, E389Q, and the double mutant E387/389Q C2I. This finding suggested that both glutamate residues, Glu387 and Glu389, are essential for the catalysis of the ADP-ribosylation by C2I. Next we studied the influence of amino acid exchanges in the region of the STS motif (Ser348-Thr349-Ser350) on transferase activity. In C2I, an additional serine residue is located at position 347. Exchange of the serine residues at position 347 and 350 with alanine had no effect on ADP-ribosylation activity. The change of both serine residues (Ser347 and Ser350) to alanine in C2I proteins resulted in a complete inhibition of the ADP-ribosyltransferase activity by about 90%. Whereas exchange of threonine to valine in position 349 caused an inhibition of the ADP-ribosyltransferase activity by about 90%, change of Ser348 with alanine resulted in a complete inhibition of the ADP-ribosyltransferase activity (Figs. 4 and 5 and Table I). Finally, we studied the functional consequences of the exchange of both arginine residues at position 299 and 300 with lysine. As shown in Fig. 4, R300K C2I catalyzed the labeling of actin. However, the enzyme activity was severely impaired.
In contrast to R300K C2I, transferase activity of the R299K mutant was not detected.

Cytotoxic Effects of the Various C2I Mutant Proteins on Cultured CHO-K1 Cells—Since site-directed mutagenesis resulted in decrease in the ADP-ribosyltransferase activity of various C2I mutants, we studied the effects of these mutants on the actin cytoskeleton of cultured cells (Fig. 6). C2I and the binding component C2II, were applied to CHO-K1 cells, growing subconfluently on coverslips. In the presence of C2II (200 ng/ml), the wild-type C2I (100 μg/ml) as well as the mutant proteins S347A C2I and S350A C2I (not shown), were capable of inducing cytotoxic effects after treatment of the cells for 1.5 h. By contrast, treatment of CHO-K1 cells with the mutant E387Q did not cause any effects on cell morphology. The mutant R300K induced a rounding up of cells after 5 h of treatment.

NAD-Glycohydrolase Activity—ADP-ribosyltransferases catalyze the hydrolysis of NAD in the absence of a protein substrate. Therefore, the C2I mutants were tested for their NAD-glycohydrolase activities (Table II). Surprisingly, the E387Q mutant of C2I, which did not show any ADP-ribosyltransferase activity, was capable of hydrolyzing NAD with a similar activity as that of the wild-type enzyme (Fig. 7). In contrast to E387Q C2I, all other mutants defective in ADP-ribosylation activity exhibited no detectable NAD-glycohydrolase activity. The quantitative analysis of the NAD-glycohydrolase activity was performed at 20 °C to minimize autohydrolysis of NAD and to determine glycohydrolase activity during the linear phase of the reaction. Under these conditions, the NAD-glycohydrolase activity of E387Q C2I was slightly but reproducibly higher than that of the wild-type C2I protein.

Photoaffinity Labeling—In order to test whether the mutations of C2I affect the binding of NAD, we used photoaffinity labeling in the presence of [carbonyl-14C]NAD. As shown in Fig. 8, UV irradiation of E387Q and of E389Q demonstrated radiolabeling of both mutant proteins. Whereas photoaffinity labeling of the C2I mutant E387Q was only slightly reduced as...
TABLE I
Specific ADP-ribosyltransferase activity of wild type and mutant C2I proteins

Activities were measured by toxin-catalyzed labeling of actin in human platelet cytosol in the presence of $^{32}$P-NAD. ADP-ribosylation was for 2.5, 5, 7.5, and 10 min at 37 °C during the linear phase of the reaction. Wild-type C2I, S347A, and S350A C2I were 10 ng/ml; R300K, T349V, and S347/350A were 100 ng/ml; all other indicated C2I mutants were used at 10 μg/ml. Enzyme activity is given as mean ± S.E. from four independent experiments. Relative enzyme activity was calculated with the activity of wild-type C2I taken as 100%.

<table>
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<th>Specific activity</th>
<th>Relative activity</th>
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<td>mmol x mol$^{-1}$ x min$^{-1}$</td>
<td>% of wild type</td>
</tr>
<tr>
<td>C2I-WT</td>
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<tr>
<td>C2I E387Q</td>
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<tr>
<td>C2I E389Q</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>C2I E387/389Q</td>
<td>&lt;0.1</td>
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<tr>
<td>C2I S347A</td>
<td>1.280 ± 240</td>
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<tr>
<td>C2I S348A</td>
<td>1.0 ± 0.2</td>
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<tr>
<td>C2I T349V</td>
<td>158 ± 42</td>
</tr>
<tr>
<td>C2I S350A</td>
<td>860 ± 230</td>
</tr>
<tr>
<td>C2I S347/350A</td>
<td>34 ± 12</td>
</tr>
<tr>
<td>C2I R299K</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>C2I R300K</td>
<td>69 ± 14</td>
</tr>
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</table>

TABLE II
Specific NAD-glycohydrolase activity of wild type and mutant C2I proteins

For determination of NAD-glycohydrolase activities, NAD hydrolysis catalyzed by the wild-type and each mutant C2I protein (100 μg/ml) was measured with 50 μM NAD for 8 h at 20 °C. Enzyme activities are given as mean ± S.E. from four independent experiments. For calculation of relative activity, the specific activity of wild type C2I was taken as 100%.

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmol x mol$^{-1}$ x h$^{-1}$</td>
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<td>C2I-WT</td>
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<tr>
<td>C2I S347A</td>
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<td>C2I R300K</td>
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* ND, not determined.

compared with wild-type C2I, the extent of radiolabeling of E389Q was decreased by about 70%. In contrast to the respective single mutants, the double mutant E387/389Q was not photoaffinity labeled.

DISCUSSION

Recently, the structural gene of the enzyme component of C. botulinum C2 toxin, C2I, was cloned and sequenced (27). Although no significant sequence identity with any other transferase was reported, we detected about 33% sequence identity of C2I with the enzyme component of iota toxin (program: PCGENE, V 6.85, 1995). After alignment of C2I with iota toxin and with various other ADP-ribosyltransferases, we assumed that the glutamic acid residue in position 389 of C2I plays a similar pivotal role for catalysis of the ADP-ribosylation reaction as Glu(S54) of iota toxin (30). Here we report that the exchange of Glu(S54) of C2I to glutamine blocked the ADP-ribosyltransferase activity as well as the NAD-glycohydrolase activity and prevented the cytotoxic effects of this toxin. These findings are in line with the notion that Glu(S54) of C2I is the catalytic glutamic acid residue that is highly conserved within the family of ADP-ribosyltransferases. Thus, Glu(S54) of C2I appears to be functionally equivalent with Glu(148) of diphtheria toxin (22), Glu(554) of Pseudomonas exotoxin A (31), Glu(128) of E. coli heat-labile enterotoxin (20), Glu(129) of pertussis toxin (32), and Glu(174) of C3 ADP-ribosyltransferase (23, 33) (see Fig. 1).

All ADP-ribosyltransferases that modify arginine residues including cholera toxin, the heat-labile E. coli enterotoxins, iota toxin, and C2 toxin contain a second glutamate residue two residues upstream of the invariant “catalytic” glutamic acid residue (34). The same holds true for some recently described eukaryotic arginine-specific ADP-ribosyltransferases (34, 35). The exchange of this second glutamic acid residue (Glu(387)) to glutamine in C2I resulted in a loss of transferase activity. This finding is in agreement with recent studies on the heat-labile E. coli toxin (36), iota toxin (30), and rabbit muscle ADP-ribosyltransferase (34). In all of these cases, the exchange of the second preceding glutamic acid residue inhibited the transferase activity. Surprisingly, the E387Q mutant of C2I still hydrolyzed NAD, indicating that this residue is essential for ADP-ribosyltransferase activity but is not required for NAD-glycohydrolase activity. In this respect, recent studies on rodent T-cell RT6 antigens, which are related to arginine-specific ADP-ribosyltransferases, are noteworthy (34, 37, 38). RT6.1, which contains the highly conserved “catalytic” glutamic acid
residue at position 209 and glutamine at position 207, demonstrates NAD-glycohydrolase activity but not transferase activity. Exchange of Glu\textsuperscript{207} of RT6.1 with glutamic acid resulted in expression of transferase activity of RT6.1 (37). In contrast, the RT6 mouse homologue RT6-1, which is characterized by an intrinsic Gln\textsuperscript{207}, possesses ADP-ribosyltransferase activity (39). Exchange of Glu\textsuperscript{207} of RT6-1 to glutamine inhibits the arginine-specific ADP-ribosyltransferase activity (37).

We performed photoaffinity labeling of the single mutants E387Q and E389Q, and of the double mutant E387Q/E389Q of C21 toxin. While the double mutant was not labeled with [carbonyl-\textsuperscript{14}C]NAD, both single mutants were radiolabeled. It has been shown with diphtheria toxin that UV irradiation cross-links NAD to the \(\gamma\)-methylene carbonyl formed by decarboxylation of the catalytic glutamic acid residue (40). We suggest that both glutamic acid residues (Glu\textsuperscript{387} and Glu\textsuperscript{389}) of C21 toxin serve as targets for UV-induced cross-linking of NAD. Thus, both glutamic acid residues seem to be located in or near the NAD-binding cleft. In line with this notion is the report on the photoaffinity labeling of iota toxin, which is also characterized by the EXE motif (Glu\textsuperscript{278}, Tyr\textsuperscript{284}–Glu\textsuperscript{286}) at the catalytic site with Glu\textsuperscript{286} being the conserved catalytic glutamic acid residue (41). In this study, UV-irradiation of iota toxin with [\textsuperscript{14}C]NAD resulted in labeling of Glu\textsuperscript{286}. Moreover, recent analysis of the crystal structure of diphtheria toxin bound to NAD and subsequent structural alignments of other ADP-ribosyltransferases suggest a close positioning of the preceding Gln/Glu residue to the active site structure.

Many mono-ADP-ribosyltransferases (e.g., pertussis toxin and \textit{E. coli} heat-labile enterotoxin) share a common STS motif about 20–70 amino acid residues upstream of the above mentioned glutamate residues (25). This motif is suggested to bind to either the ribose or the phosphate of the AMP moiety of NAD (42). In C21, the STS motif is extended to SSTS (Ser\textsuperscript{347} through Ser\textsuperscript{350}). Exchange of the first (Ser\textsuperscript{347}) or the last (Ser\textsuperscript{350}) serine residues of this sequence to alanine, did not change transferase activity substantially. Similarly, the exchange of both Ser\textsuperscript{347} and Ser\textsuperscript{350} to alanine reduced but did not eliminate transferase activity. These findings support the notion that neither serine residue Ser\textsuperscript{347} or Ser\textsuperscript{350} is essential for catalysis. Whereas exchange of Thr\textsuperscript{349} with valine reduced the enzyme activity, the exchange of Ser\textsuperscript{348} to alanine eliminated transferase activity. Thus, Ser\textsuperscript{348} may play an essential role in NAD-binding or catalysis. In pertussis toxin and \textit{E. coli} heat-labile enterotoxin, the first serine residue of the STS motif overlaps with Tyr\textsuperscript{344} of diphtheria toxin and most likely participates in formation of a groove in which the nicotinamide ring and NMN ribose of NAD fit (42).

An important role in transferase activity is ascribed to the arginine residues at positions 9 and 7 of pertussis toxin (43) and \textit{E. coli} heat-labile toxin (36), respectively. These arginine residues overlap exactly with His\textsuperscript{351} of diphtheria toxin, an amino acid residue, which is essential for transferase activity although it may not directly be involved in catalysis (44). Deductions from crystal analysis (20, 42) have suggested that these arginine/histidine residues are essential for NAD-binding and/or maintenance of the active-site integrity of the ADP-ribosyltransferases. We tested whether Arg\textsuperscript{299} or Arg\textsuperscript{300} plays a similar crucial role in C21 toxin activity. Only the exchange of Arg\textsuperscript{299} to alanine induced a dramatic reduction in transferase activity. Therefore we propose that the arginine at position 299 is equivalent to Arg\textsuperscript{7} of \textit{E. coli} heat-labile toxin and Arg\textsuperscript{9} of pertussis toxin. Moreover, our findings are in full agreement with recent site-directed mutagenesis of iota toxin showing that only Arg\textsuperscript{295}, and not Arg\textsuperscript{296}, is essential for transferase activity (30).

In summary, we demonstrate that Glu\textsuperscript{389} represents the highly conserved catalytic glutamic acid residue, and Glu\textsuperscript{387} is essential for transferase but not for NAD-glycohydrolase activity. Furthermore, we identify that Ser\textsuperscript{348} is essential for transferase activity. Finally, our studies suggest that Arg\textsuperscript{299} of C21 toxin and not Arg\textsuperscript{300} is the conserved arginine residue which is most likely involved in NAD binding and/or stabilization of the active site structure.

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Holger Barth, Jan C. Preiss, Fred Hofmann and Klaus Aktories

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