Active Site Mutation of the C3-like ADP-Ribosyltransferase from *Clostridium limosum*—Analysis of Glutamic Acid 174[†,⊥]

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ABSTRACT: *Clostridium limosum* ADP-ribosyltransferase modifies low molecular mass GTP-binding proteins of the Rho subtype family. Here we cloned and sequenced the gene of the transferase and expressed it in *Escherichia coli*. The gene encodes a protein of 250 amino acids (M_r = 27 840), with a putative signal peptide of 45 amino acids, that shows about 60−65% identity with C3 transferases from *Clostridium botulinum*. The mature *C. limosum* transferase was expressed as a maltose-binding fusion protein in *E. coli* and purified to apparent homogeneity. To study the functional role of Glu174 of *C. limosum* transferase, which was recently photoaffinity-labeled with [*carbonyl*-^{14}C]NAD [Jung, M., et al. (1993) *J. Biol. Chem. 268,* 23215−23218], two mutants E174D and E174Q were constructed by a polymerase chain reaction-based system. The E174D and E174Q mutants showed a dramatic decrease in k_{cat}, but no major changes in $K_{m,NAD}$. Furthermore, replacement of Glu174 by aspartic acid and glutamine largely reduced and completely blocked UV-induced incorporation of [*carbonyl*-^{14}C]NAD into the transferase. The data indicate that Glu174 is an active site residue of *C. limosum* transferase.

A family of bacterial ADP-ribosyltransferases selectively modifies low molecular mass GTP-binding proteins of the Rho subtype family. Members of this enzyme family are *Clostridium botulinum* C3 exoenzyme (Aktories et al., 1987, 1988), *Clostridium limosum* transferase (Just et al., 1992), *Bacillus cereus* transferase (Just et al., 1995), and a transferase called EDIN[1] (Inoue et al., 1991), which is produced by certain strains of *Staphylococcus aureus*. Moreover, several isoforms of C3 have been reported that are 60−70% identical on the amino acid level. EDIN is only about 35% identical with C3 and shows no immunological cross-reactivity (Inoue et al., 1991). All of these transferases have molecular masses of about 25−28 kDa, are rather basic proteins (p*I* >9), and ADP-ribosylate the low molecular mass GTP-binding proteins RhoA, -B, and -C (Braun et al., 1989; Chardin et al., 1989; Sugai et al., 1992). ADP-ribosylation of Rho occurs at Asn41 (Sekine et al., 1989), a modification that renders the target protein biologically inactive (Paterson et al., 1990).

Recently, we labeled the transferase from *C. limosum* in the presence of [*carbonyl*-^{14}C]NAD by UV irradiation and identified the acceptor amino acid of the label by protein chemistry in a conserved sequence at the C-terminus, which corresponds to position 174 of *C. botulinum* exoenzyme C3 (Jung et al., 1993). To compare *C. limosum* transferase with C3 and to study the functional role of the radiolabeled glutamic acid residue in more detail, we then sequenced the *C. limosum* transferase gene and constructed mutants. Here we report that the selective changes in the recently photoaffinity-labeled glutamic acid residue (Glu174) to Asp or Gln reduced the transferase activity by more than 1000-fold without major changes in the binding of NAD. These findings indicate that Glu174 is part of the active site of *C. limosum* transferase.

EXPERIMENTAL PROCEDURES

Materials. *C. limosum* ADP-ribosyltransferase were purified as described (Just et al., 1992). Recombinant RhoA (Paterson et al., 1990) and Rac1 (Ménard et al., 1992) proteins were purified as described. Endoprotease Lys-C was obtained from Boehringer (Mannheim, Germany). [*adenyl*-^{32}P]NAD and [*carbonyl*-^{14}C]NAD were from Dupont NEN (Bad Homburg, Germany). Nucleotides were from Boehringer. All other reagents were from Sigma (Deisenhofen, Germany).

Bacterial Strains and Vectors. *C. limosum* strain 2 (Just et al., 1992) was used as the source of *C. limosum* ADP-ribosyltransferase and of DNA for cloning the exoenzyme gene. A *C. limosum* ADP-ribosyltransferase-specific library was constructed in pUC19 (Biolabs, Germany) and pBluescript (Stratagene, Heidelberg, Germany) vectors using TG1. Plasmid pMal-c2 (Biolabs, Schwalbach, Germany) was used for expression of the *C. limosum* 2 ADP-ribosyltransferase gene in TG1.

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[⊥] The nucleotide sequence for the *C. limosum* C3 gene has been submitted to the EMBL Nucleotide Sequence Database under accession number X87215.

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[1] Abbreviations: C3, *Clostridium botulinum* ADP-ribosyltransferase C3; DTT, dithiothreitol; EDIN, epidermal differentiation inhibitor from *Staphylococcus aureus*; Lys-C, endoprotease Lys-C; pBlue, pBluescript; MAP, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reactions; SDS, sodium dodecyl sulfate; WT, wild-type.

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Gene Cloning and Expression. All procedures were according to Maniatis et al. (1982), unless otherwise specified. Restriction endonucleases, factor Xa, and Klenow polymerase were from Biolabs and other enzymes were from Boehringer. DNA was ^{32}P-labeled by a Random-Prime-It-Kit (Stratagene) and sequenced by the didesoxy chain termination procedure using a Sequenase Kit (USB, Cleveland, OH). Competent *E. coli* cells were prepared by the method of Hanahan (1985). Oligonucleotide primers used for polymerase chain reactions and DNA sequencing were purchased from MWG-Biotech (Ebersberg, Germany).

Isolation of DNA from C. limosum. The method was adapted from that of Nemoto et al. (1991) used for the isolation of DNA from *C. botulinum* C strain 003-9. *C. limosum* 2 was grown in a modified cooked meat medium (Just et al., 1992) under anaerobic conditions for 2 days at 37 °C. The cells were harvested by centrifugation at 5000*g* for 15 min and suspended in 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.15 M NaCl. Lysozyme (2 mg/mL) was added, and after incubation for 4 h at 37 °C, the EDTA concentration was raised to 0.1 M and sarcosin and proteinase K were added to final concentrations of 1% (w/v) and 10 mg/mL, respectively. The mixture was incubated overnight at 37 °C. Resultant lysate was successively extracted twice with phenol, phenol/chloroform (1:1), and chloroform, and the aqueous phase was treated with 50 μg/mL RNAse A for 60 min at 37 °C. The mixture was extracted twice with phenol, phenol/chloroform (1:1), and chloroform, and DNA was precipitated with ethanol and resuspended in TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA] at a concentration of 1 mg/mL.

Southern Blot Analysis. *C. limosum* DNA cleaved with restriction enzymes was electrophoresed on a 0.4% agarose gel (10 μg of DNA/lane) and transferred to Nylon membranes (Hybond-N, Amersham) as described by Southern (1975). Filters were prehybridized in 7% sodium dodecyl sulfate (SDS), 1 mM EDTA (pH 8.0), 0.5 M Na_2HPO_4 (pH 7.2), and 1% bovine serum albumin for 2 h at 60 °C. Hybridization was performed in the same solution containing 10^6–10^7 cpm/mL ^{32}P-labeled DNA probe (see the following) overnight at 60 °C. Filters were washed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% SDS, and 1 mM EDTA (pH 8.0) and, subsequently, in 4×, 2×, and 1× SSC, 0.5% SDS, and 1 mM EDTA (pH 8.0) at 60 °C. The membranes were exposed to Kodak X-OMAT films for 2 days.

Construction and Screening of the C. limosum ADP-Ribosyltransferase-Specific Library. For isolation of the complete gene encoding for *C. limosum* ADP-ribosyltransferase, two different genomic libraries were constructed. First, *C. limosum* DNA was digested with *Pst*I and electrophoresed on a 0.4% agarose gel. A DNA fragment of 1.6 kb hybridized with the ^{32}P-labeled 0.6 kb *Hind*III fragment from pMRP36 (Popoff et al., 1991), a pKK233-2 vector haboring the DNA region coding for the mature C3 transferase protein. DNA from the 1.3−1.9 kb region was eluted, purified, and cloned into the *Pst*I site of pUC19. Recombinant clones were screened by colony hybridization using the same probe as before. Plasmid DNA from a positive clone (pUC19-2.1 plasmid) was isolated and sequenced. In a second approach, *C. limosum* DNA was digested with *Rsa*I. A 0.8 kb DNA fragment hybridized with a ^{32}P-labeled 328 bp *Hinc*II fragment from the 3′-part of the *C. limosum* transferase gene (pUC19-2.1). DNA from the 0.7−1.0 kb region was eluted and cloned into a *Bam*HI site of pBluescript-II KS± via *Bam*HI adapters. Recombinant clones were screened by colony hybridization with the *Hinc*II fragment described earlier. pBluescript plasmid containing the insert at the *Bam*HI site (pBlue-2.91 plasmid) was isolated and sequenced.

Construction of Plasmid for the Expression of C. limosum ADP-Ribosyltransferase in E. coli TG1. Because both clones isolated (pUC19-2.1; pBlue-2.91) did not contain the complete coding region of *C. limosum* transferase gene, but contained 5′- and 3′-regions that overlapped, the complete transferase gene was constructed via *Pst*I sites in pBluescript (pBlue-WT). To delete the upstream and downstream noncoding regions (including the signal peptide) (see Results), polymerase chain reactions were performed to amplify the gene encoding for the mature *C. limosum* exoenzyme. Primers used for the amplification of the *C. limosum* ADP-ribosyltransferase gene contained *Bam*HI and *Eco*RI restriction sites, which allowed directed cloning in the prokaryotic expression vector pMal-c2. Proper construction was confirmed by DNA sequencing of the resultant plasmid (pMal-c2-WT).

Construction of Mutants. Commercially available T3 primer and mutant *C. limosum* 42-mer oligonucleotides (E174D: 5′-AAC CTA TTA GTA CCT TTA AAG GTC AAC TT**G** **AC**G TGT TGC TTC-3′) (E174Q: 5′- AAC CTA TTA GTA CCT TTA AAG GTC AAC TT**C** **AA**G TGT TGC TTC-3′) were used for the amplification of the 3′-part of the *C. limosum* ADP-ribosyltransferase gene by polymerase chain reactions (PCR). Polymerase chain reactions were performed according to Maniatis et al. (1989) in a 100 μL reaction volume of 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 2.5 units of Taq DNA polymerase, 100 pmol of primers, and 10 ng of pBlue-WT1 with annealing at 42 °C and 25 cycles. Reaction products were resolved on a 0.7% agarose gel. The PCR fragments of 1300 bp were eluted from the gel and cloned into the *Hinc*II pUC19-2.1 site. Proper construction and mutations were confirmed by DNA sequencing of the resulting plasmids. Construction of the complete mutant gene was performed under essentially the same conditions as described for the wild-type toxin.

Protein Preparations from E. coli TG1. *E. coli* TG1 cells carrying the appropriate fusion plasmids (pMal-c2-WT, pMalc2-E174D, and pMalc2-E174Q) were grown at 37 °C in LB medium containing 100 μg/mL ampicillin until OD_{600} = 0.5. Expression of recombinant *C. limosum* transferase was induced by isopropyl β-D-thiogalactopyranoside (IPTG) added to a final concentration of 100 μg/mL, and the cells were grown for an additional 2.5 h. Cells were harvested by centrifugation at 5000*g* for 15 min. The cell pellet was resuspended in 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 1 mM EDTA and sonicated three times for 1 min at 0 °C. Cell debris remaining and intact cells were removed by centrifugation at 12000*g* for 20 min. The fusion protein was purified by affinity chromatography and cleaved by factor Xa according to the manufacturer's instructions (Biolabs). The protein was subjected to DEAE-A50 chromatography (1.5 × 10 cm column, flow rate 1 mL/min) and recovered in the flow-through. Finally, maltose was separated by a NAP-5 column. Purified enzymes were lyophilized and resuspended in 50 mM Tris-HCl (pH 7.5).

ADP-Ribosylation Assay. The ADP-ribosylation reaction was performed essentially as described (Just et al., 1992). Recombinant RhoA or Rac1 (0.3 μg each) was incubated in medium (100 μL) containing 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 50 mM Tris-HCl (pH 7.5), and 10 μM [^{32}P]-NAD (about 1 μCi) or as indicated for 15 min at 37 °C. For kinetic studies, five NAD concentrations between 1 and 100 μM were used, and the incubation times for wild-type and mutant transferases were 4 and 30 min, respectively. This incubation time covered the linear phase of the reaction. The specific activities of the transferases were also studied in the presence of 5 μM [^{32}P]NAD for up to 3 h at 25 °C. ^{32}P-labeled proteins were analyzed by SDS−PAGE according to Laemmli (1970), with subsequent autoradiography or phosphorimaging (Molecular Dynamics). The amount of incorporated ADP−ribose was calculated as nanomoles $(minute)^{-1}$ $(milligram)^{-1}$.

NAD-Glycohydrolase Activity Assay. For the determination of the NAD-glycohydrolase activity, the recombinant proteins (100 μg/mL WT, 1 mg/mL E174D, and 1 mg/mL E174Q) were incubated in medium (50 μL) containing 10 mM triethanolamine hydrochloride (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT, and 100 μM [^{32}P]NAD for 4 h at 37 °C. Aliquots (5 μL) of the reaction mixture were separated by TLC (Silica gel 60, Merck, Germany) with isopropyl alcohol (66%)/1% ammonium sulfate (33%). Quantitative analyses of ADP−ribose and NAD were performed by phosphorimaging (Molecular Dynamics). The amount of ADP−ribose formed was calculated as picomoles $(minute)^{-1}$ $(milligram)^{-1}$ (±SE).

Immunoblot Analysis. Immunoblotting was performed according to Towbin et al. (1979) with anti-C3 IgG (1:10 000). The second antibody was anti-rabbit IgG coupled to peroxidase, and visualization was performed with the ECL system from Amersham.

Fluorescence Quenching. The decrease in intrinsic protein fluorescence of WT, E174D, and E174Q as a function of the NAD^+ concentration was measured at 25 °C. Solutions of the proteins [in 2 mL of Tris-HCl (20 mM, pH 7.5)] were excited at 295 nm (4 nm band pass), and the fluorescence intensity was measured over the range 315−400 nm (10 nm band pass) in a Perkin-Elmer luminescence spectrometer (LS 50). The final concentration of the proteins used in the experiments was 0.5 μM. The fluorescence decrease of L-tryptophan as a function of NAD concentration was used to correct the inner filter effect. The dissociation constant (K_d) was calculated by regression analysis of the changes in fluorescence intensity versus the NAD^+ concentration.

Protease Digestion. The relative stabilities of the mutant proteins were assessed by proteolytic digestion with endoprotease Lys-C. In parallel reactions, each toxin (15 μg) was incubated without and with 2 μg of Lys-C in a total volume of 100 μL of 50 mM Tricine-HCl (pH 8.0) and 10 mM EDTA for 12 h at 30 °C. Reactions were stopped by the addition of sample buffer and heating for 5 min at 95 °C. Thereafter, partially digested proteins were analyzed by SDS−PAGE and Coomassie Blue staining.

Photolabeling with [carbonyl-^{14}C]NAD. Photoaffinity labeling was performed essentially as described (Jung et al., 1993). Transferases (2 nmol) were incubated in 125 μL of 25 mM ammonium bicarbonate (pH 7.3) and 2 mM $MgCl_2$ with [*carbony*-^{14}C]NAD (20 nmol) for 1 h on a microtiter plate at 4 °C. UV irradiation (154 nm, 3 cm distance, 3000 $\mu W/cm^2$) was performed at 4 °C for up to 210 min. At the indicated time points, 10 μL aliquots were removed and diluted with triethanolamine hydrochloride (10 mM, pH 7.5). Thereafter, the amount of labeling was determined by precipitation of photolabeled protein by trichloroacetic acid (30%, w/v) and subsequent filtration onto nitrocellulose filters. The filters were washed with 15 mL of 6% (w/v) trichloroacetic acid, and the remaining activity was counted by liquid scintillation.

Protein Concentration. Protein concentration was determined according to Bradford (1976) with bovine serum albumin as standard.

RESULTS

Recent photoaffinity studies on the active site structure of *C. limosum* transferase were based on partial amino acid sequencing of the clostridial enzyme (Jung et al., 1993). To gain more insight into the structure of the transferase and to compare its primary sequence to that of *C. botulinum* C3 ADP-ribosyltransferase, we cloned and sequenced the DNA of the *C. limosum* transferase as described under Experimental Procedures. Because both enzymes are immunologically related (Just et al., 1992), we used a DNA probe of C3 strain C 468, which corresponded to the 0.6 kb *Hind*II fragment from the C3 pMRP36 vector (Popoff et al., 1991) for Southern analysis of *C. limosum* DNA and screening of a *Pst*I library in pUC 19. Sequencing of the insert DNA of the positive clone (pUC19-2.1) revealed that the cloned fragment was largely homologous to the 3′-part of the C3 gene. This DNA fragment was then used for Southern analysis of *Rsa*I-digested *C. limosum* DNA and screening of a *Bam*HI library in pBluescript. Sequencing of a positive pBluescript insert (pBlue-2.91) revealed a DNA fragment largely homologous to the 5′-part of the C3 gene.

Figure 1A shows the sequence of the complete gene of *C. limosum* obtained from pBlue-2.92 and pUC19-2.1. The gene contains an open reading frame of 750 bp, starting with ATG (nucleotides 154−156) and terminating with TAA (nucleotides 905−907), encoding for a protein of 250 amino acids with a M_r of 27 840. By comparison with the C3 transferases from *C. botulinum* type C strain 468 (Popoff et al., 1991), we suggest that the encoded protein consists of a signal sequence of 45 amino acid residues and the mature protein starting after Lys45 with Pro46. The deduced amino acid sequence matches peptides Asn34−Arg41, Ile46−Arg51, Ser74−Lys77, Met78−Arg88, Glu129−Ala144, Ala160−Ile166, and Ala160−Ala179 (mature protein numbering) of *C. limosum* ADP-ribosyltransferase determined by protein chemistry (Just et al., 1992, 1993). The nucleotide sequence of *C. limosum* transferase was identical to the sequences of C3 ADP-ribosyltransferase reported by Popoff et al. (1991) and Nemoto et al. (1991) by about 71% and 74%, respectively. On an amino acid level, *C. limosum* transferase was identical with C3 by about 60% (Popoff et al., 1991) and 65% (Nemoto et al., 1991) (Figure 1B). The glutamic acid residue, which was recently photoaffinity-labeled with [*carbonyl*-^{14}C]NAD in *C. limosum* transferase, was localized at position 174 of the mature protein.

To test the functional role of Glu174, we changed the glutamic acid residue to glutamine and to aspartic acid. For expression of the proteins in *E. coli*, we used the prokaryotic expression vector pMal-c2. As shown in Figure 2A, the

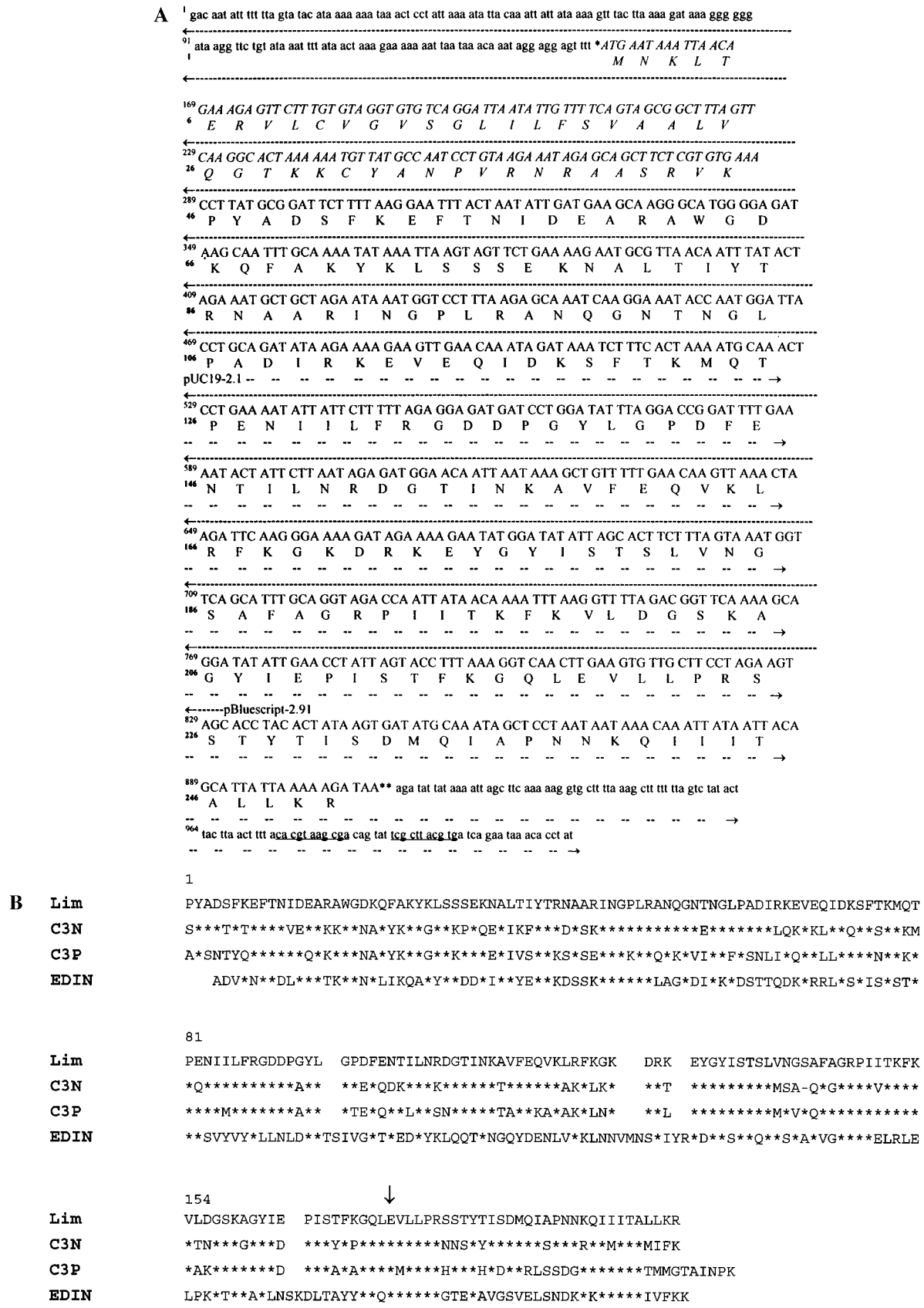

FIGURE 1: (A) Nucleotide sequence of the gene for *C. limosum* transferase and predicted amino acid sequence. The nucleotide sequences of a 1.6 kbp *Pst*I fragment in pUC29-2.1 (dashed line) and of a 0.8 kbp *Rsa*I fragment in pBlue-2.91 (solid line) were determined. Nucleotides of the noncoding and coding regions are given in lower case and capital letters, respectively. Amino acids of the proposed signal sequence are indicated by italic letters. The underlined region is a putative transcription terminator. The nucleotide sequence reported has been submitted to the EMBL Nucleotide Sequence Database under accession number X87215 (*C. limosum* C3 gene). (B) Comparison of the primary structure of the *C. limosum* transferase (Lim) with *C. botulinum* C3 transferases (C3N, C3P) and EDIN. * indicates identical amino acid; an arrow indicates glutamic acid 174. Amino acid sequences of C3N, C3P, and EDIN are cited from the following references: Nemoto et al. (1991), Popoff et al. (1991), and Inoue et al. (1991), respectively.

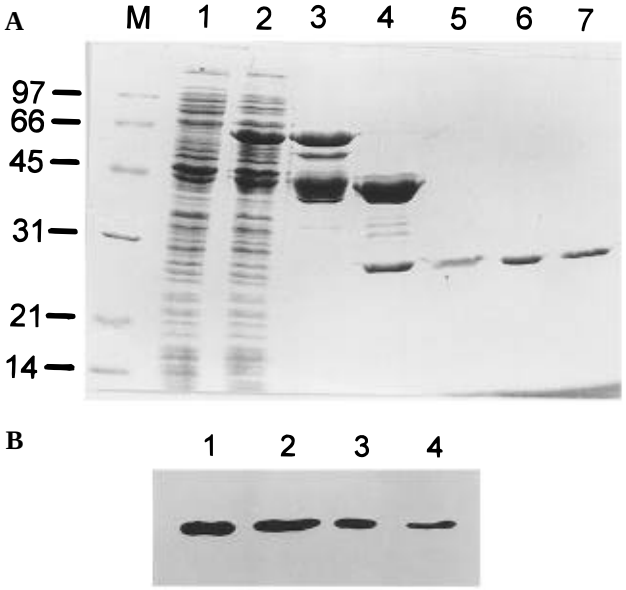

FIGURE 2: (A) Purification of *C. limosum* ADP-ribosyltransferase from maltose-binding protein (MBP)−transferase fusion protein. Lane assignments: lane 1, control *E. coli* lysate; lane 2, *E. coli* lysate after induction of the MBP−transferase (WT) fusion protein; lane 3, eluate from the amylose matrix; lane 4, amylose eluate after treatment with factor Xa; lane 5, flow-through from DEAE column, wild-type transferase; lane 6, DEAE flow-through, E174D mutant transferase; lane 7, DEAE flow-through, E174Q mutant transferase; M, M_r marker. (B) Immunoblots of *C. limosum* transferase and recombinant ADP-ribosyltransferases. Anti-C3 antibody (IgG) (1: 10 000) cross-reacted with recombinant WT (lane 1), E174D (lane 2), E174Q (lane 3), and *C. limosum* transferase (lane 4). Each lane contained 1 μg of protein.

mature recombinant wild-type and mutant (E174D, E174Q) *C. limosum* transferases were expressed and purified to apparent homogeneity by amylose affinity chromatography, factor Xa cleavage, and an additional DEAE anion exchange chromatography. Polyclonal antibody generated toward *C. limosum* transferase cross-reacted with the purified recombinant proteins (Figure 2B). Moreover, the Lys-C digestion pattern of wild-type and mutant *C. limosum* transferases indicated similar protease susceptibility (not shown).

Next we studied the enzyme activity of the transferases. As found for the ADP-ribosyltransferase from *C. limosum* (Just et al., 1992), the recombinant transferases selectively modified recombinant Rho but not Rac proteins (not shown). With recombinant RhoA as protein substrate, wild-type transferase exhibited a k_{cat} value of 0.28 min^{-1}, a K_m value for NAD of about 52 μM, and a catalytic efficiency of 5.3 $\times 10^3$ M^{-1} min^{-1} (Table 1). The high K_m for NAD obtained with recombinant proteins was surprising, because the $K_{m,NAD}$ of *C. limosum* transferase was about 0.5 μM by using crude platelet membrane preparations, as reported recently (Just et al., 1992). The enzyme activity of the E174D mutant was reduced by about 1200-fold, whereas the K_m for NAD of the E174D mutant was only increased from about 52 to about 102 μM. Because the enzyme activity of the E174Q mutant was even lower than that of the E174D mutant transferase, determination of k_{cat} and K_m for NAD was impossible under these conditions. However, we compared the enzyme activities in the presence of rather low concentrations of NAD (5 μM) at 25 °C (Figure 3). As deduced from these data, the relative specific activities of the E174D and E174Q mutant transferases were 1×10^{-3} and 1.3×10^{-4}, respectively, relative to that of the wild-type enzyme. Mutation of Glu174 induced a dramatic reduction in NAD glycohydrolase activity. Whereas K_m and k_{cat} of the wild-type enzyme were determined to be 160 μM and 2×10^{-3} min^{-1}, respectively, the glycohydrolase activities of both mutants (E714D, E1174Q) were below our detection limit. Similar reductions in ADP-ribosyltransferase activity of the E174D and E174Q mutants were obtained with recombinant C3 ADP-ribosyltransferase. For these studies, however, recombinant C3 transferase mutants were used as a crude *E. coli* extract (not shown).

Table 1: Kinetics of ADP-Ribosyltransferase Activity[a]

	WT	E174D	E174Q
K_m (μM)	52	102	ND
v_{max} (nmol/min/mg)	12	0.014	ND
relative v_{max}	1	1.2×10^{-3}	ND
k_{cat} (1/min)	0.28	3.2×10^{-4}	ND
relative k_{cat}	1	1.2×10^{-3}	ND
k_{cat}/K_m (M^{-1} min^{-1})	5.3×10^3	3.2	ND
relative k_{cat}/K_m	1	5.7×10^{-4}	ND

[a] Assays were performed as described under Experimental Procedures. The kinetic parameters were derived from regression analysis. NAD concentrations were 5−100 μM for WT and 10−250 μM for E174D. Final transferase concentrations were 1 μg/mL WT, 10 μg/mL E174D, and 10 μg/mL E174Q. Incubation time was 4 min for WT and 30 min for E174D and E174Q at 37 °C. ND, not detectable. Standard deviation was not greater than ±15%.

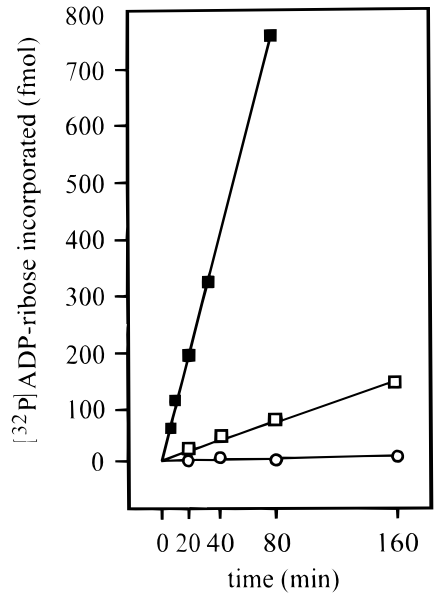

FIGURE 3: Time course of ADP-ribosylation of RhoA by recombinant WT and mutant (E174D and E174Q) *C. limosum* transferases. WT (0.1 μg/mL, ■), E174D (10 μg/mL, □), and E174Q (10 μg/mL, ○) transferases were incubated in the presence of 1 μg of RhoA and 5 μM [^{32}P]NAD for the indicated times at 25 °C. Thereafter, labeled Rho protein was analyzed by SDS−PAGE and phosphorimaging.

To confirm that the mutation has no major effects on NAD binding, we studied the affinity of NAD for *C. limosum* transferase by quenching of the intrinsic protein fluorescence. As shown in Figure 4, quenching of intrinsic protein fluorescence for WT, E174D, and E174Q by NAD was similar. The percent of quenching at each given NAD concentration was calculated from the data and analyzed by Scatchard plot to determine the dissociation constants (K_d) for the wild-type and mutant transferases. Surprisingly, the

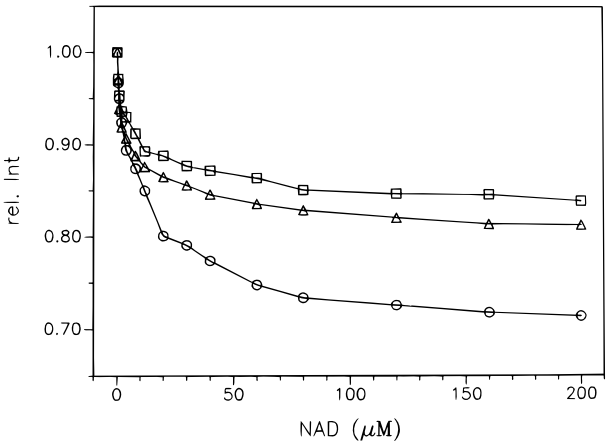

FIGURE 4: Quenching of intrinsic protein fluorescence by NAD for recombinant *C. limosum* transferase WT (○), E174D (□), and E174Q (△). Data were corrected for NAD self-quenching, and the percent quenching at each NAD concentration was calculated from the data and analyzed by Scatchard plots to determine the dissociation constants for the wild-type and mutant proteins. K_d values obtained are summarized in Table 2.

Table 2: Comparison of Dissociation Constants of WT, E174D, and E174Q

protein	λ_{max}[a] (nm)	K_{d1}(NAD)[b] (μM)	K_{d2}(NAD)[b] (μM)
WT	335	2.0	25
E174D	338	1.6	50
E174Q	340	1.0	40

[a] Determined as the maximal fluorescence emission over the range 310−400 nm, with excitation at 295 nm. [b] Determined from Scatchard analysis of percent quenching as a function of NAD concentration (see also Figure 4). Standard deviation was not greater than ±15%.

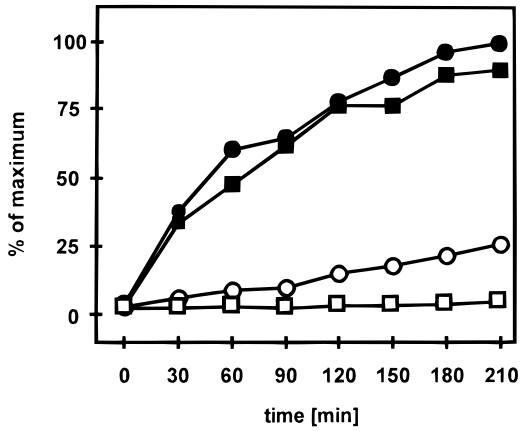

FIGURE 5: Photoaffinity labeling of wild-type and mutant transferases in the presence of [*carbonyl*-^{14}C]NAD. Native *C. limosum* (●), wild-type (■), and E174D (○) and E174Q (□) mutant transferases were UV-irradiated for the indicated times in the presence of [*carbonyl*-^{14}C]NAD. Thereafter, the ^{14}C radiolabel incorporated was determined as described under Experimental Procedures. Maximal incorporation of the radiolabel with native *C. limosum* transferase (~0.7 mol/mol) was set to 100%.

data were best fit by the assumption of two NAD-binding sites showing K_d values of 1−2 and 25−50 μM, respectively (Table 2).

Finally, we studied the photoaffinity labeling of recombinant wild-type and mutant transferases in comparison to the transferase produced by *C. limosum*. As shown in Figure 5, after UV irradiation in the presence of [*carbonyl*-^{14}C]NAD, recombinant wild-type transferase incorporated the same amount of ^{14}C label as native transferase from *C. limosum*. In contrast, the incorporation of the aspartic acid mutant was largely reduced (~80%), and no incorporation of label was detected with the glutamine mutant.

DISCUSSION

Recently, we reported on the photolabeling of a glutamic acid residue of *C. limosum* ADP-ribosyltransferase by UV irradiation in the presence of [*carbonyl*-^{14}C]NAD (Jung et al., 1993). From the finding that photolabeling resulted in the inhibition of ADP-ribosyltransferase and glycohydrolase activity, we concluded that the glutamic acid residue was located at the active site of the transferase. To obtain direct evidence for involvement of the glutamic acid residue in catalysis, we applied site-directed mutagenesis. To this end, the *C. limosum* transferase gene was first cloned and sequenced. The transferase gene from *C. limosum* was very similar to that for C3 ADP-ribosyltransferase from *C. botulinum* types C and D with about 60−64% identity. The calculated molecular weight of the transferase was 27 840. The glutamic acid residue, which was recently photoaffinity-labeled by [*carbonyl*-^{14}C]NAD (Jung et al., 1993), was identified to be located at position 174 of the mature *C. limosum* transferase. In consideration of recent findings that *C. botulinum* types C and D produce two types of ADP-ribosyltransferases (Moriishi et al., 1993), the position of this glutamic acid residue is the same in C3 from *C. botulinum* strain 468 (Popoff et al., 1991) and is localized at position 173 in C3 from *C. botulinum* strain 0003-9 (Nemoto et al., 1991).

Studies on the enzyme activities of wild-type and mutant transferases indicate that Glu174 is part of the active site of C3-like enzymes. For example, change of Glu174 to Asp or Gln reduced the k_{cat} of the enzyme by more than 1000-fold. Importantly, this mutation did not cause a comparable reduction in NAD binding. This was studied by enzyme kinetics and by protein fluorescence quenching. Previous kinetic studies with *C. limosum* transferase performed with cell membrane preparations revealed K_m values of 0.3−0.5 μM. Similar results were obtained with other C3-like enzymes (Just et al., 1992). Moriishi reported $K_{m,NAD}$ values of 50 and 150 nM determined with C3 isoforms and rat brain membranes (Moriishi et al., 1993). In contrast, the enzyme kinetics with recombinant RhoA as substrate resulted in K_m values of about 52 μM. Moreover, we found, by fluorescence quenching with NAD, two binding sites with apparent high and low affinity for NAD. The reason for this discrepancy from earlier studies is not clear. It might be that conformational changes of the transferase induced by lipids from the membrane preparations are responsible for changes in the NAD-binding sites and for the different K_m values. In this respect it is noteworthy that low concentrations of sodium dodecyl sulfate were shown to reduce the $K_{m,NAD}$ for the ADP-ribosylation of recombinant RhoA by C3 transferase by about 10-fold (Just et al., 1993). Nevertheless, our studies indicate that the mutations did not cause major changes in the affinity for NAD, which could explain the decrease in enzyme activity.

Thus, the kinetic data from mutant enzyme indicate that Glu174 is the active site residue, which is equivalent to Glu148 of diphtheria toxin (Carroll & Collier, 1984; Wilson et al., 1990), Glu553 of *Pseudomonas* exotoxin A (Carroll & Collier, 1987), and Glu129 of pertussis toxin (Antoine et

al., 1993; Barbieri et al., 1989). In all of these cases, mutation of the active site glutamic acid residue by aspartic acid resulted in a several hundred fold reduction in enzyme activity (Wilson et al., 1990; Douglas & Collier, 1987). Moreover, replacement of the critical glutamic acid (Glu148 of diphtheria toxin) by glutamine resulted in a further reduction in transferase activity. The same held true for *C. limosum* transferase. The E174Q mutant exhibited less transferase activity than the E174D mutant. These effects most likely were not due to gross changes in the overall structure of the transferase, because NAD binding and susceptibility toward endoprotease Lys-C were not changed. Mutations of Glu174 in *C. limosum* transferase largely reduced the glycohydrolase activity to values below our detection limit. This finding is similar to one for pertussis toxin, where changes in the active site Glu129 cause strong inhibition in glycohydrolase activity (Locht et al., 1989). In contrast, mutation of Glu148 of diphtheria toxin substantially reduced transferase activity without major effects on the hydrolase activity (Wilson et al., 1990).

ADP-ribosyltransferases from *B. cereus* (Just et al., 1995) and *S. aureus* (EDIN) (Inoue et al., 1991) have been reported to ADP-ribosylate Rho proteins. These transferases are more distantly related to C3 and do not show immunological cross-reactivity with clostridial transferases. However, the catalytic region that exhibits high sequence homology appears to be conserved (Just et al., 1995; Inoue et al., 1991). Interestingly, a glycosylphosphatidylinositol-anchored ADP-ribosyltransferase from rabbit skeletal muscle actin (Takada et al., 1995) and a NAD glycohydrolase RT6.2 (Takada et al., 1994) show considerable sequence homology to the region adjacent to Glu174 of C3-like transferases. In fact, when Glu240 of rabbit skeletal muscle transferase, which is suggested to be equivalent to Glu174 of *C. limosum* transferase, was changed to Asp, the transferase activity was reduced by about 250-fold, indicating a pivotal role in catalysis (Takada et al., 1995).

Photoaffinity labeling with *C. limosum* transferase recombinant wild-type and mutant enzymes revealed that Glu174 is the only amino acid residue that is modified after UV irradiation in the presence of [*carbonyl*-^{14}C]NAD. Even a small reduction in the amino acid length (from glutamic acid to aspartic acid) causes a dramatic decrease in labeling, which reflects a large reduction in enzyme activity. Similar effects were obtained by photoaffinity labeling of diphtheria toxin and its mutants (Wilson et al., 1990). These findings suggest that the cosubstrate NAD and the active site glutamic acid residues exhibit the same orientation and most likely the same atomic distances in the catalytic pocket of *C. limosum* transferase, diphtheria toxin, and perhaps various other ADP-ribosylating toxins.

So far the precise role of the critical glutamic acid residue is still unclear. The findings that the conservative exchange of Glu174 with Asp largely reduced transferase activity and that exchange with Gln blocked enzyme activity indicate that both the chain length of the carboxyl group and the negative charge of Glu174 are critical with respect to its catalytic function. Similar results were obtained for Glu148 of diphtheria toxin (Wilson et al., 1990), Glu553 of *Pseudomonas* exotoxin A (Douglas & Collier, 1987), and Glu129 of pertussis toxin (Locht et al., 1989). It was proposed that the essential carboxylate of the glutamic acid residue serves as a general base to increase the nucleophilicity of the incoming protein substrate to attack the nicotinamide−ribose bond of NAD (Wilson & Collier, 1992). On the other hand, it has been suggested that the action of the critical glutamic acid is primarily on NAD (Takada et al., 1994, 1995; Locht et al., 1989; Antoine et al., 1993). This notion takes into consideration the fact that various ADP-ribosyltransferases share very similar active site structures with respect to NAD and glutamic acid, whereas the enzymes use different amino acid accepters (e.g., diphthamide by diphtheria toxin, arginine by cholera toxin, cysteine by pertussis toxin, and asparagine by C3-like toxins) [for a review, see Aktories and Just (1993)]. Moreover, recent findings that the conserved region around Glu174 of C3-like toxins is also present in the NAD glcyohydrolase RT6.2 (Takada et al., 1994) support the view (Takada et al., 1995) that glutamic acid acts on NAD rather than on a protein substrate.

Taken together, our studies provide direct evidence for the involvement of Glu174 in the catalytic activity of *C. limosum* ADP-ribosyltransferase, confirm a recent report (Jung et al., 1993) on UV photoaffinity labeling of this amino acid residue in the presence of NAD, and, finally, corroborate our hypothesis (Jung et al., 1993) that this amino acid residue is equivalent to the previously identified active site glutamic acids from diphtheria toxin, *Pseudomonas* exotoxin A, and pertussis toxin.

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