Identification of Glutamic Acid 381 as a Candidate Active Site Residue of *Pseudomonas aeruginosa* Exoenzyme S[†]

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ABSTRACT: Exoenzyme S of *Pseudomonas aeruginosa* (ExoS) is a member of the family of bacterial ADP-ribosylating exotoxins (bAREs). Site-directed mutagenesis of glutamic acids within the catalytic domain of ExoS (termed ΔN222) allowed the identification of the preferential inactivation of ADP-ribosyltransferase activity by alanine substitution of E381. The specific activity of the E381A mutant was 0.02% of wild-type ΔN222. ΔN222(E381A) retained the requirement of factor activating exoenzyme S (FAS) activation for the expression of ADP-ribosyltransferase activity. In contrast, E387A, E399A, and E414A mutants possessed ADP-ribosyltransferase activity similar to that of wild-type ΔN222. Kinetic evaluation of E381A and two other mutants, E381D and E381S, showed that their primary defect was a lower k_{cat} in the ADP-ribosylation of soybean trypsin inhibitor (SBTI). The K_m for NAD and SBTI and activation by FAS varied 2- and 10-fold relative to ΔN222. In addition, the E381 mutants possessed identical protease patterns during thrombin and trypsin digestion as ΔN222, which indicated that E381 mutants had retained their overall conformation. Together, these data identify E381 as contributing to the catalytic activity of exoenzyme S.

Pseudomonas aeruginosa produces two ADP-ribosyltransferases, exotoxin A (ETA)[1] and exoenzyme S (Iglewski et al., 1978). ETA and exoenzyme S differ with respect to the eukaryotic proteins that are targeted for ADP-ribosylation (Coburn et al., 1989a,b, 1991) and by the requirement of exoenzyme S for a eukaryotic accessory protein, termed *f*actor *a*ctivating exoenzyme *S* (FAS), to express ADP-ribosyltransferase activity *in vitro* (Coburn et al., 1991). The gene encoding FAS was cloned from a bovine brain cDNA library and shown to be a member of the 14-3-3 family of eukaryotic proteins (Fu et al., 1993). Although the protein targeted for ADP-ribosylation *in vivo* has not been defined, exoenzyme S has been shown to ADP-ribosylate a number of eukaryotic proteins *in vitro*, including Ras and several other small molecular weight GTP-binding proteins, vimentin, and SBTI (Coburn et al., 1989). SBTI is a convenient target to measure the *in vitro* ADP-ribosyltransferase activity of exoenzyme S.

Exoenzyme S has been implicated as a virulence determinant of *P. aeruginosa* which contributes to pathogenesis in burn wound and chronic lung infection models (Nicas & Iglewski, 1984). Exoenzyme S was purified from the culture supernatant fluid of *P. aeruginosa* strain 388 as an aggregate which contained both a 53-kDa protein and a 49-kDa protein [discussed in Kulich et al. (1993)]. The 49-kDa form of exoenzyme S possessed enzymatic activity following elution from sodium dodecyl sulfate (SDS)−polyacrylamide gels (Coburn et al., 1991; Nicas & Iglewski, 1984) and has been designated the enzymatically active form of exoenzyme S. The 53-kDa form of exoenzyme S possessed little apparent ADP-ribosyltransferase activity *in vitro* (Nicas & Iglewski, 1984). Recent studies have shown that the 53-kDa and 49-kDa forms of exoenzyme S were encoded by separate genes (Kulich et al., 1995).

The gene encoding the 49-kDa form of exoenzyme S (*exoS*) has been cloned (Kulich et al., 1994). ExoS (453 amino acids) has been expressed in an enzymatically active form in *Escherichia coli*, which showed that ExoS was necessary and sufficient for the expression of FAS-dependent ADP-ribosyltransferase activity. Deletion mapping experiments showed that the carboxyl-terminal 222 of ExoS represented the FAS-dependent ADP-ribosyltransferase domain (Knight et al., 1995). In the present study, alanine-scanning mutagenesis was used to identify E381 as a candidate active site glutamic acid residue of ExoS.

EXPERIMENTAL PROCEDURES

Materials

E. coli TG1 and BL21 (DE3) were obtained from Amersham and Novagen, respectively. Dideoxy-DNA sequencing was performed with a kit from Promega. Oligonucleotide-directed mutagenesis was performed with a kit from Amersham Corp. Oligonucleotides were synthesized by either GIBCO/BRL or OPERON. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. [*adenylate phosphate*-^{32}P] NAD was purchased from Dupont-New England Nuclear and [*nicotinamide*-^{3}H] NAD was purchased from Amersham. Ni^{2+} affinity resin and pET15b vector were purchased from Novagen. Recombinant FAS was a gift from Drs. H. Fu

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[1] Abbreviations: bAREs, bacterial ADP-ribosylating exotoxins; ΔN222, carboxyl-terminal 222 amino acids of exoenzyme S; SBTI, soybean trypsin inhibitor; FAS, factor activating exoenzymes; ETA, *Pseudomonas aeruginosa* exotoxin A; CT/LT, cholera toxin and heat-labile enterotoxin of *Escherichia coli*; TCA, trichloroacetic acid; pET, plasmid-containing T7 promoter expression system.

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(Emory University) and J. Collier (Harvard Medical School). Bovine serum albumin (BSA) was purchased from Pierce Biochemicals. Trypsin, SBTI, and thrombin were purchased from Sigma.

Methods

Site-Directed Mutagenesis. DNA encoding the carboxyl-terminal 222 amino acids of ExoS (ΔN222) (Knight et al., 1995) was cloned into M13 and used as template for mutagenesis, using the oligonucleotide-directed *in vitro* mutagenesis Sculptor System (Amersham). Mutagenesis was performed essentially as described by the manufacturer. Several mutations were introduced into ΔN222. Residues within ΔN222 are designated with respect to the predicted amino acid sequence of ExoS (Kulich et al., 1994), using the single-letter designation of the wild-type amino acid at a specific residue followed by the single amino acid designation of the substituted amino acid. Oligonucleotides (complement of the coding strand) used in this study were (mutated bases are underlined) E381A, 5′-ATAGAGAA-5′-ATAGAGAATAGCTTTTTCAT-3′; E381S, 5′-ATAG-AGAATCGATTTTTCATTC-3′; E381D, 5′-TAGAGAAT GTCTTTTTCAT-3′; E387A, 5′-CATGTCGGTAGCTTT-GTTAT-3′; E399A, 5′-CACTCCCTGAGCATCGCTGG-3′; and E414A, 5′-GCCACTCTGAGCCCCCAGGG-3′. Mutated DNA was subjected to sequence analysis to identify the presence of desired base change(s) and confirm that no secondary mutations were present. The *Xba*I−*Bam*HI DNA fragment containing the mutated ΔN222 gene was isolated from the Rf of M13 and inserted into the *Xba*I−*Bam*HI digested pET15b vector to yield the respective pET15bΔN222 mutant. The pET15bΔN222 mutant was transformed into *E. coli* TG1 and then into *E. coli* BL21 (DE3) for protein expression. ΔN222 and ΔN222 mutants were expressed as fusion proteins in the pET expression system, which introduced 6-histidine amino acids at the amino terminus of ΔN222.

Protein Purification. ΔN222 and ΔN222 mutants were purified from *E. coli* BL21 (DE3), using Ni^{2+} affinity chromatography under conditions previously described (Knight et al., 1995). Briefly, overnight cultures of *E. coli* containing the appropriate pET15b vector were diluted 1/50 into 800 mL of L-broth which contained 100 μg of ampicillin/mL and incubated at 30 °C with shaking. After 2 h, IPTG was added to a final concentration of 0.6 mM, and the cultures were incubated for an additional 2 h. Cultures were centrifuged at 6000*g* for 10 min, the cell pellet was suspended in binding buffer (20 mM Tris-HCl, pH 7.9, containing 500 mM NaCl and 5 mM imidazole), and then 12 μg of DNase I/mL, 12 μg of RNase A/mL, 24 μg of leupeptin/mL in EtOH, 12 μg of aprotinin/mL in H_2O, and 1.2 mM PMSF in EtOH (final concentrations) were added. Cells were broken with a French press, and extracts were centrifuged at 30000*g* for 12 min; the soluble material was passed through a 0.45 μm cellulose nitrate filter and then subjected to Ni^{2+} affinity chromatography (2 mL column, Novagen). The column was washed with 20 mL of binding buffer and then with 20 mL of binding buffer containing 25 mM imidazole. ΔN222 and ΔN222 mutants were eluted with binding buffer containing 250 mM imidazole. Fractions (1 mL) which contained ΔN222 or ΔN222 mutants were pooled and analyzed by SDS−PAGE and Coomassie blue staining. The 6-histidine tag was not removed from the fusion proteins, since earlier studies showed that this tag did not influence the ADP-ribosyltransferase activity of ExoS (Knight et al., 1995).

ADP-ribosylation of SBTI. Linear Velocity Assay. Reaction mixtures contained in 25 μL 0.2 M sodium acetate (pH 6.0), 30 μM [*adenylate phosphate*-^{32}P] NAD (specific activity 1 Ci/mmol), 60 μM SBTI, 0.25 μM recombinant FAS, and an aliquot of ΔN222 or ΔN222 mutant which had been diluted in 25 mM Tris (pH 7.6) containing 0.1 mg of egg albumin/mL. In the linear velocity assays, 0.1 pmol of ΔN222 equivalents was added to the reaction mixture. Assays were performed at room temperature, and 20 μL aliquots were spotted onto trichloroacetic acid (TCA) saturated Whatman 3MM chromatography paper at the indicated time points: 2.5, 5, and 10 min for ΔN222, ΔN222-(E387A), ΔN222(E399A), and ΔN222(E414A); 1, 2, and 3 h for ΔN222(E381A) and ΔN222(E381S); and 10, 20, and 40 min for ΔN222(E381D). Papers were washed twice for 30 min with 7.5% TCA and once with methanol and dried. Radioactivity was detected by scintillation counting.

Kinetic Analysis. *(a) Variable SBTI*. Dilutions of SBTI were made in 10 mM Tris (pH 7.6) containing 0.1 mg of egg albumin/mL. The final concentration of SBTI was varied from 19.5 to 333 μM for ΔN222 and 29 to 750 μM for ΔN222(E381A), ΔN222(E381S), and ΔN222(E381D). Other components in the reaction mixture were as described in the velocity assay above. At the appropriate time [10 min for ΔN222, 20 min for ΔN222(E381D), and 3 h for ΔN222-(E381A) and ΔN222(E381S)], an aliquot (20 μL) was removed and spotted onto TCA-saturated paper. The amount of SBTI utilized was less than 10%. Data were transformed to the Lineweaver−Burk equation with the assistance of Enzfitter (Elsevier, Cambridge, U.K.).

(b) Variable NAD. The concentration of NAD was varied from 19.5 and 333 μM for ΔN222 and from 29 to 750 μM for ΔN222(E381A), ΔN222(E381S), and ΔN222(E381D). Assays were performed as described in (a) above. The utilization of NAD was less than 10%.

Gel-Filtration Chromatography. ΔN222 and ΔN222-(E381A), ΔN222(E381S), and ΔN222(E381D) were subjected to Sephacryl-200HR gel filtration [100 mL column equilibrated in 10 mM Tris (pH 7.6) containing 20 mM NaCl]. Column fractions were subjected to SDS−PAGE followed by Coomassie blue staining.

Proteolysis of Proteins. ΔN222 and ΔN222 mutants were purified by Ni^{2+} affinity chromatography followed by gel filtration and subjected to proteolysis. Thrombin digestion: Reaction mixtures contained in 40 μL 50 mM sodium citrate (pH, 6.5), 6.4 μg of ΔN222 or ΔN222 mutants, and 0.2 μg of thrombin and were incubated at room temperature for 15, 30, and 60 min. Aliquots (10 μL) were analyzed by SDS−PAGE. Trypsin digestion: Reaction mixtures contained in 40 μL 25 mM Tris-HCl (pH 8.0), 0.1 mM $CaCl_2$, 6.4 μg of ΔN222 or ΔN222 mutants, and 0.08 μg of trypsin. Analysis was performed as described above for thrombin.

Protein Quantitation. ΔN222 and ΔN222 mutants were subjected to SDS−PAGE, and the Coomassie-stained bands were quantitated by densitometry, using an AMBIS optical-imaging system, and normalized to a known concentration of BSA, except gel-filtration purified proteins which were quantitated spectrophotometrically, using BSA as a standard.

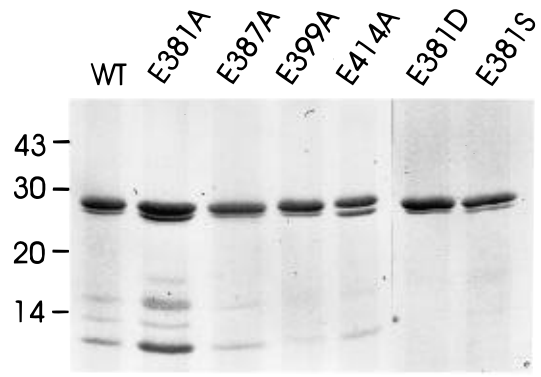

FIGURE 1: SDS−PAGE analysis of purified ΔN222 and ΔN222 mutants. ΔN222 and ΔN222 mutants were purified by Ni^{2+} affinity chromatography and subjected to reduced 13.5% SDS−PAGE followed by Coomassie blue staining. Purified proteins are indicated above their respective lanes. Migration of molecular mass markers (kDa) is indicated in the left margin.

RESULTS

Expression and Purification of ΔN222 and ΔN222 Mutants. Initial alignments of the predicted amino acid sequence of ExoS with other bAREs identified E265, E408, or E409 as potential active site glutamic acids of ExoS (Kulich et al., 1994; Kulich and Barbieri, unpublished data). However, mutants of ExoS that possessed conserved amino acid substitutions at either E265, E408, or E409 had wild-type levels of ADP-ribosyltransferase activity (Kulich and Barbieri, unpublished data). Subsequent deletion mapping identified the carboxyl-terminal 222 amino acids of ExoS as its ADP-ribosyltransferase domain (Knight et al., 1995). This deletion protein was termed ΔN222 and used in the present study. To determine whether ExoS possessed an active site glutamic acid, as observed for other bAREs, several additional glutamic acid residues were targeted for mutational analysis. There are 17 glutamic acid residues within ΔN222; glutamic acid residues were chosen for analysis due to their location within the primary amino acid sequence of ExoS with respect to the S343-T344-S345 sequence of ExoS. Although the biochemical function of this S-T-S sequence has not been resolved, it is conserved within all known bAREs which ADP-ribosylate either small-molecular-weight or heterotrimeric G-proteins (Kulich et al., 1994). In the case of pertussis toxin and cholera toxin, the active site glutamic acid lies carboxyl terminal to the S-T-S sequence (Krueger & Barbieri, 1994). Thus, E381, E387, E399, and E414 were changed to alanine, and the mutated proteins were analyzed for ADP-ribosyltransferase activity.

ΔN222 and ΔN222 mutants were expressed in *E. coli* BL21 (DE3), using the pET expression system, as histidine-fusion proteins composed of a 22 amino acid amino-terminal sequence, conferred by the vector, fused to ΔN222. Ni^{2+} affinity purified ΔN222 and ΔN222 mutants possessed apparent molecular masses of 26 kDa as determined by SDS−PAGE at similar purities which were >80% (Figure 1). When stored at 4 °C, degradation was detected; note the degradation in the ΔN222(E381A) preparation which became apparent following its storage for 1−2 weeks at 4 °C; the purity of a freshly prepared sample of ΔN222-(E381A) is shown in Figure 2. Proteins were stable for several months when stored in 40% glycerol at −20 °C (data not shown). Enzymatic analyses were performed on freshly prepared protein preparations.

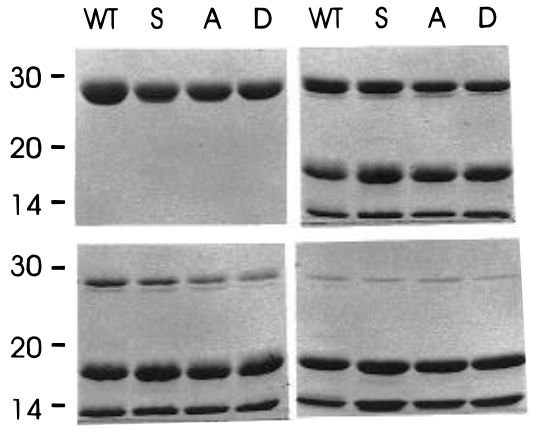

FIGURE 2: Thrombin digestion of ΔN222, ΔN222(E381A), ΔN222-(E381D), and ΔN222(E381S). Proteins (6.4 μg) were incubated with 0.2 μg of thrombin and at the indicated times (time; 0, upper left panel; 15 min, upper right panel; 30 min, lower left panel; and 60 min, lower right panel) subjected to SDS−PAGE followed by Coomassie staining. Proteins are indicated above their respective lanes.

Table 1: ADP-ribosylation of SBTI by ΔN222 and ΔN222 Mutants

	ADP-ribosyltransferase activity[a]		
enzyme	specific activity[b]	relative activity (%)[c]	FAS dependent[d]
ΔN222	152.6 ± 41	100	+
E381A	0.03 ± 0.01	0.02	+
E381D	0.9 ± 0.3	0.6	+
E381S	0.02 ± 0.003	0.01	+
E387A	46.6 ± 16	31	+
E399A	42.9 ± 16	28	+
E414A	30.6 ± 22	20	+

[a] Enzyme assays were performed as described under Experimental Procedures. [b] Specific activity was expressed as picomoles of ADP-ribose incorporated into SBTI per minute per picomole of enzyme. [c] Activities were expressed relative to ΔN222 which was normalized to 100%. [d] + indicates that expression of ADP-ribosyltransferase activity required the presence of FAS.

ADP-ribosyltransferase and NAD Glycohydrolase Activities of ΔN222 and ΔN222 Mutants. ADP-ribosyltransferase activity was determined as the incorporation of [*adenylate phosphate*-^{32}P] NAD into SBTI. Under linear velocity conditions, ΔN222(E381A) possessed 0.02% of ADP-ribosyltransferase activity of ΔN222 (Table 1), while the activities of ΔN222(E387A), ΔN222(E399A), and ΔN222-(E414A) approached ΔN222. Two additional mutations were introduced at residue 381 where glutamic acid was changed to an aspartic acid or serine residue. The E381D mutation addressed whether the alignment of the carboxyl group was important for ADP-ribosylation, while the E381S mutation addressed the need for the carboxylic acid and the potential role of hydrogen bonding in the ADP-ribosylation reaction. Under linear velocity conditions, the specific activity of E381D was 0.6% of ΔN222 and the specific activity of E381S was 0.01% of ΔN222 (Table 1). All ΔN222 mutants tested in this study required the presence of FAS to express ADP-ribosyltransferase activity.

ΔN222 catalyzed the NAD glycohydrolyase at a specific activity of 2.8 ± 0.4 mol of nicotinamide released min^{-1} (mol of ΔN222)$^{-1}$. Expression of NAD glycohydrolase activity by ΔN222 was FAS dependent. In contrast, ΔN222-(E381A), at the same concentration as of ΔN222, did not

Table 2: Kinetic Constants for ΔN222 and ΔN222 Mutants in the ADP-ribosylation of SBTI[a]

enzyme	K_m (μM)	k_{cat} (pmol min^{-1} pmol^{-1})	k_{cat}/K_m
variable NAD			
ΔN222	40 ± 9	308 ± 45	7.7
E381A	206 ± 28	0.2 ± 0.1	0.001
E381D	224 ± 77	5.8 ± 1.4	0.03
E381S	134 ± 16	0.09 ± 0.01	0.0007
variable SBTI			
ΔN222	49 ± 20	216 ± 63	4.4
E381A	429 ± 146	0.1 ± 0.04	0.0002
E381D	387 ± 28	5.1 ± 1.0	0.013
E381S	230 ± 92	0.04 ± 0.009	0.0002

[a] ADP-ribosyltransferase activity was measured as described in Methods to variable NAD or SBTI.

catalyze detectable NAD glycohydrolase activity. NAD glycohydrolase activity was assayed as previously described (Cortina & Barbieri, 1991). The inability of ΔN222(E381A) to catalyze either ADP-ribosyltransferase or NAD glycohydrolase activities is similar to the catalytic properties of E129 mutants of pertussis toxin (Pizza et al., 1988) but differed from diphtheria toxin E148 mutants which expressed reduced ADP-ribosyltransferase activity with wild-type amounts of NAD glycohydrolase activity (Wilson, 1990).

Kinetic Characteristics of ΔN222, ΔN222(E381A), ΔN222-(E381D), and ΔN222(E381S). K_m and k_{cat} values were established for ΔN222(E381A), ΔN222(E381D), and ΔN222-(E381S) to determine a role for E381 in the ADP-ribosylation reaction. Initial linear velocities, at variable SBTI and NAD, could be transformed to a Lineweaver−Burk plot, which indicated that both ΔN222 and ΔN222 mutants followed Michaelis−Menten kinetics. At variable NAD (Table 2), the K_m apparent for NAD was 40 μM for ΔN222, 206 μM for E381A, 224 μM for E381D, and 134 μM for E381S. The k_{cat} value decreased about 1500-fold for E381A, 53-fold for E381D, and 3400-fold for E381S, with respect to wild-type ΔN222. The calculated k_{cat}/K_m ratio was 7.7 for ΔN222, 0.001 for E381A, 0.03 for E381D, and 0.0007 for E381S. At variable SBTI (Table 2), the K_m apparent for SBTI was 50 μM for ΔN222, 400 μM for E381A and E381D, and 200 μM for E381S. The k_{cat} value decreased 2100-fold for E381A, 42-fold for E381D, and 5400-fold for E381S, with respect to wild-type ΔN222. The calculated k_{cat}/K_m ratio was 4.4 for ΔN222, 0.0002 for E381A, 0.013 for E381D, and 0.0002 for E381S.

Interaction of ΔN222 and ΔN222(E381A) with FAS. To determine whether mutations at residue 381 affected the activation efficiency by FAS, ADP-ribosyltransferase activity was measured under linear velocity conditions as a function of FAS. A 6-fold molar increase in FAS (between 3- and 9-fold molar excess of FAS) yielded 5% and 10% increases in the ADP-ribosyltransferase activity of ΔN222 and ΔN222-(E381A), respectively (data not shown). These results suggested that ΔN222 and ΔN222(E381A) had similar affinities for FAS.

Protease Sensitivity of ΔN222, ΔN222(E381A), ΔN222-(E381D), and ΔN222(E381S). The stability and conformational differences among ΔN222, ΔN222(E381A), ΔN222-(E381D), and ΔN222(E381S) were measured as a function of protease susceptibility, using thrombin and trypsin. There are two predicted thrombin binding sites within ΔN222, one within the amino terminus encoded by the vector and a second defined by the sequence ^{319}**RG**T**RG**^{323}, where either bolded RG could represent a thrombin recognition site. Proteins were incubated with thrombin and subjected to SDS−PAGE. As shown in Figure 2, ΔN222 and ΔN222 mutants were degraded at similar rates sequentially through the two thrombin sites, which indicated that the accessibility of these sites was similar among the four proteins. The observed sizes of the thrombin fragments were consistent with thrombin cleavage within the ^{319}**RG**T**RG**^{323} sequence. Trypsin digestions were also performed on ΔN222 and ΔN222 mutants. Although the proteolytic fragments generated by trypsin were more complex, the tryptic digests were identical among the four proteins (data not shown). These data, along with the observation that the binding affinities for NAD, SBTI, and FAS had not dramatically changed, suggested that residue 381 plays a catalytic role in the ADP-ribosyltransferase activity of ExoS.

DISCUSSION

A growing number of bacteria produce exotoxins that catalyze the transfer of ADP-ribose from NAD to eukaryotic target proteins (Collier & Mekalanos, 1980; Jacobson & Jacobson, 1989; Krueger & Barbieri, 1994). Although members of the family of bAREs differ with respect to their quaternary structure and lack primary amino acid homology, bAREs possess several conserved properties. Crystallographic studies have shown that the active sites of LT and ETA (Sixma et al., 1991) and DT and ETA (Choe et al., 1992) are essentially superimposable. In the case of LT and ETA, their active sites were observed to superimpose despite the conservation of only 3 of the 44 amino acids within this region. In addition to structural similarities, bAREs possess a conserved glutamic acid which functions in catalysis (Wilson et al., 1990; Antoine et al., 1993; Antoine & Locht, 1994). The presence of an active site glutamic acid in DT and other bAREs provided the catalyst to search for an active site glutamic acid within ExoS.

Although FASTA analysis of the predicted amino acid sequence of the 49-kDa form of exoenzyme S (ExoS) failed to align ExoS with other members of the family of bAREs, local primary amino acid homology between ExoS and sequences of the active site of LT/CT were observed. One alignment predicted that E265 of ExoS and E112 of LT/CT were homologues. This alignment was of particular interest, since E112 represented the catalytic active site glutamic acid of CT/LT as determined by both structural and molecular biological analysis (Lobet et al., 1991; Sixma et al., 1991). The prediction of a similar conserved catalytic glutamic acid between ExoS and LT/CT was consistent with these two proteins being the only known members of the family of bAREs that required a eukaryotic accessory protein to express ADP-ribosyltransferase activity (Coburn et al., 1991). Subsequent alignments also identified E408 and E409 of ExoS as possible homologues to E112 of CT/LT (Kulich and Barbieri, unpublished data). Attempts to confirm a role for either E265, E408, or E409 of ExoS in the ADP-ribosyltransferase reaction showed that amino acid substitutions at either E265, E408, or E409 failed to modify enzymatic activity. Thus, a second strategy was undertaken to determine whether ExoS possessed an active site glutamic acid, where several glutamic acids were chosen for mutagenesis with respect to the S-T-S within ΔN222 (Kulich et al., 1994). In other bacterial ADP-ribosyltransferases active site

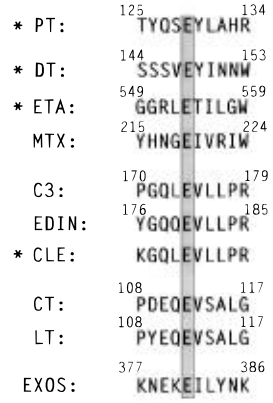

FIGURE 3: Alignment of active site glutamic acids within the family of bAREs. An asterisk (*) denotes those bAREs that have been shown to possess an active site glutamic acid via photolabeling with NAD (Carroll & Collier, 1984). The abbreviations used are as follows: PT, pertussis toxin; DT, diphtheria toxin; ETA, *P. aeruginosa* exotoxin A; MTX, *Bacillus sphaericus* mosquitocidal toxin; C3, *Clostridium botulinum* C3 exoenzyme; EDIN, *Staphylococcus aureus* strain E-1 epidermal differentiation inhibitor; CLE, *Clostridium limosum* exoenzyme; CT, cholera toxin; LT, *E. coli* heat-labile enterotoxin; and EXOS, *P. aeruginosa* exoenzyme S.

glutamic acids lie carboxyl terminal to the S-T-S sequence [reviewed in Krueger and Barbieri (1994)].

Linear velocity analysis showed that N222(E381A) catalyzed FAS-dependent ADP-ribosylation of SBTI at about a 3000-fold slower rate than wild-type ΔN222. Kinetic analysis of several E381 mutants showed that the primary effect of substitutions at E381 was to reduce the k_{cat} in the ADP-ribosyltransferase reaction. The most conservative substitution tested was aspartic acid which reduced the k_{cat} by about 50-fold, while both the alanine and serine substitutions yielded several thousandfold reductions to the k_{cat}. The K_ms for both NAD and SBTI varied between 2- and 10-fold for each of the mutants tested. Similar substitutions for the active site glutamic acids of DT(E148) or PT-S1(E129) yielded similar reductions to the k_{cat} of the ADP-ribosylation reaction but smaller reductions in the affinity for NAD and target protein (Wilson et al., 1990; Antoine et al., 1993; Antoine & Locht, 1994). Thus, E381 may not only contribute to catalysis but also be located within a region of the active site that contributes to structural integrity. It appears that the perturbation to ligand binding occurred via a local mechanism which did not perturb overall protein structure, since proteolysis experiments did not detect changes to either thrombin or trypsin sites within the E381 mutants.

Figure 3 shows the alignment of the known active site glutamic acids of the bAREs. Although there is little consensus primary amino acid conservation immediately surrounding the active site glutamic acids, bAREs can be aligned into subgroups according to the target protein that is ADP-ribosylated. This alignment shows that the primary amino acid sequence surrounding E381 is unique to even the C3 subgroup of bAREs. Like ExoS, C3 has been shown to ADP-ribosylate a subset of small-molecular-weight GTP-binding proteins, including Rho (Jung et al., 1993). Thus, ExoS may represent a new subset within the bARE family. Studies are currently underway to determine the contribution of the 53-kDa form of exoenzyme S in ADP-ribosyltransferase activity and to determine whether the 53-kDa form of exoenzyme S possesses an E381 homologue.

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REFERENCES

Antoine, R., & Locht, C. (1994) *J. Biol. Chem. 269*, 6450−6457.
Antoine, R., Tallett, A., van Heyningen, S., & Locht, C. (1993) *J. Biol. Chem. 268*, 24149−24155.
Carroll, S. F., & Collier, R. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3307−3311.
Choe, S., Bennett, M. J., Fujii, G., Curmi, P. M. G., Kantardjieff, K. A., Collier, R. J., & Eisenberg, D. (1992) *Nature 357*, 216−222.
Coburn, J., & Gill, D. M. (1991) *Infect. Immun. 59*, 4259−4262.
Coburn, J., Dillon, T. S., Iglewski, B. H., & Gill, D. M. (1989a) *Infect. Immun. 57*, 996−998.
Coburn, J., Wyatt, R. T., Iglewski, B. H., & Gill, D. M. (1989b) *J. Biol. Chem. 264*, 9004−9008.
Coburn, J., Kane, A. V., Feig, L., & Gill, D. M. (1991) *J. Biol. Chem. 266*, 6438−6446.
Collier, R. J., & Mekalanos, J. J. (1980) in *Multifunctional Proteins* (Bisswanger, H., & Schmincke-Ott, E., Eds.) pp 261−291, John Wiley and Sons, New York.
Cortina, G., & Barbieri, J. T. (1989) *J. Biol. Chem. 264*, 17322−17328.
Fu, H., Coburn, J., & Collier, R. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2320−2324.
Iglewski, B. H., Sadoff, J., Bjorn, M. J., & Maxwell, M. S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3211−3215.
Jacobson, M. K., & Jacobson, E. L. (1989) *ADP-ribose Transfer Reactions, Mechanisms and Biological Significance*, Springer, New York.
Jung, M., Just, I., van Damme, J., Vandekerckhove, J., & Aktories, K. (1993) *J. Biol. Chem. 267*, 10274−10280.
Knight, D. A., Finck-Barbançon, V., Kulich, S. M., & Barbieri, J. T. (1995) *Infect. Immun. 63*, 3709−3711.
Kulich, S. M., Yahr, T., Mende-Mueller, L., Barbieri, J. T., & Frank, D. W. (1994) *J. Biol. Chem. 269*, 10431−10437.
Kulich, S. M., Frank, D. W., & Barbieri, J. T. (1995) *Infect. Immun. 63*, 1−8.
Krueger, K. M., & Barbieri, J. T. (1994) *Clin. Microbiol. Rev. 8*, 34−47.
Lobet, Y., Cluff, C. W., & Cieplak, W. (1991) *Infect. Immun. 59*, 2870−2879.
Nicas, T. I., & Iglewski, B. H. (1984) *Infect. Immun. 45*, 470−474.
Pizza, M., Bartoloni, A., Prugnola, A., Silvestri, S., & Rappuoli, R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7521−7525.
Sixma, T. K., Pronk, S. E., Kalk, K. H., Wartna, E. S., van Zanten, B. A. M., Witholt, B., & Hol, W. G. J. (1991) *Nature 351*, 371−377.
Wilson, B. A., Reich, L. A., Weinstein, B. R., & Collier, J. R. (1990) *Biochemistry 29*, 8643−8651.

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