JOURNAL OF BACTERIOLOGY, Mar. 1997, p. 1609–1613 Vol. 179, No. 5
0021-9193/97/$04.00+0

Biochemical Relationships between the 53-Kilodalton (Exo53) and 49-Kilodalton (ExoS) Forms of Exoenzyme S of *Pseudomonas aeruginosa*

SUYAN LIU, TIMOTHY L. YAHR, DARA W. FRANK, AND JOSEPH T. BARBIERI*

Department of Microbiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Received 4 October 1996/Accepted 26 December 1996

Genetic studies have shown that the 53-kDa (Exo53) and 49-kDa (ExoS) forms of exoenzyme S of *Pseudomonas aeruginosa* are encoded by separate genes, termed *exoT* and *exoS*, respectively. Although ExoS and Exo53 possess 76% primary amino acid homology, Exo53 has been shown to express ADP-ribosyltransferase activity at about 0.2% of the specific activity of ExoS. The mechanism for the lower ADP-ribosyltransferase activity of Exo53 relative to ExoS was analyzed by using a recombinant deletion protein which contained the catalytic domain of Exo53, comprising its 223 carboxyl-terminal residues (termed N223-53). N223-53 was expressed in *Escherichia coli* as a stable, soluble fusion protein which was purified to >80% homogeneity. Under linear velocity conditions, N223-53 catalyzed the FAS (for factor activating exoenzyme S)-dependent ADP-ribosylation of soybean trypsin inhibitor (SBTI) at 0.4% and of the Ras protein at 1.0% of the rates of catalysis by N222-49. N222-49 is a protein comprising the 222 carboxyl-terminal residues of ExoS, which represent its catalytic domain. N223-53 possessed binding affinities for NAD and SBTI similar to those of N222-49 (less than fivefold differences in K_ms) but showed a lower velocity rate for the ADP-ribosylation of SBTI. This indicated that the primary defect for ADP-ribosylation by Exo53 resided within its catalytic capacity. Analysis of hybrid proteins, composed of reciprocal halves of N223-53 and N222-49, localized the catalytic defect to residues between positions 235 and 349 of N223-53. E385 was also identified as a potential active site residue of Exo53.

Exoenzyme S is an ADP-ribosyltransferase produced by *Pseudomonas aeruginosa* (9). Exoenzyme S purifies from spent culture medium as an aggregate enriched for two proteins with apparent molecular masses of 53 and 49 kDa, which were designated the 53- and 49-kDa forms of exoenzyme S, respectively. The 49-kDa form of exoenzyme S possessed ADP-ribosyltransferase activity following elution from sodium dodecyl sulfate (SDS)-polyacrylamide gels (3, 19) and was designated the enzymatically active form of this exoenzyme. In contrast, the 53-kDa form of exoenzyme S possessed little detectable ADP-ribosyltransferase activity in vitro (19). Exoenzyme S has been implicated as a virulence determinant of *P. aeruginosa* in burn wound and chronic lung infection models (19).

Exoenzyme S is a member of the family of bacterial ADP-ribosylating exotoxins (for a review, see reference 13). Coburn and Gill showed that exoenzyme S required a eukaryotic accessory protein, termed FAS (for factor activating exoenzyme S), to catalyze the ADP-ribosyltransferase reaction in vitro (3). The gene encoding FAS has been cloned from a bovine brain cDNA library and shown to be a member of the 14-3-3 family of eukaryotic proteins (8). Although the protein targeted for ADP-ribosylation in vivo has not been defined, exoenzyme S can ADP-ribosylate several eukaryotic proteins in vitro, including Ras, vimentin, and soybean trypsin inhibitor (SBTI) (4). SBTI has been used as a convenient target protein for biochemical studies.

The 53- and 49-kDa forms of exoenzyme S are encoded by separate genes (15, 22). The gene encoding the 49-kDa form of exoenzyme S (*exoS*) was cloned from a cosmid library of *P. aeruginosa* 388 chromosomal DNA (16). Recombinant forms of ExoS (453 amino acids) expressed in *Escherichia coli* possessed FAS-dependent ADP-ribosyltransferase activity (15). This showed that ExoS is necessary and sufficient for expression of FAS-dependent ADP-ribosyltransferase activity. Deletion mapping subsequently localized the FAS-dependent ADP-ribosyltransferase activity to the carboxyl-terminal 222 amino acids of ExoS (12). The 53-kDa form of exoenzyme S (*exoT*) has also been cloned (22). Recombinant forms of Exo53 (457 amino acids) were shown to catalyze the FAS-dependent ADP-ribosyltransferase reaction at a specific activity that was about 0.2% of that of ExoS. Alignment of the primary amino acid sequences of ExoS and Exo53 showed that there is 76% primary amino acid homology (22) but did not allow a prediction of the mechanism responsible for the disparate catalytic activities of the two proteins. In this study, we define the biochemical defect of Exo53.

MATERIALS AND METHODS

Materials. *E. coli* TG1 and BL21(DE3) were obtained from Amersham and Novagen, respectively. Dideoxy-DNA sequencing was performed with a Pharmacia automated DNA sequencer. Oligonucleotide-directed mutagenesis was performed with a kit from Amersham Corp. Oligonucleotides were synthesized at the Protein-Nucleic Acid Shared Facility at the Medical College of Wisconsin. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. [^{32}P-adenylate phosphate]NAD was purchased from Dupont-New England Nuclear, and [^{3}H-nicotinamide]NAD was purchased from Amersham. Ni^{2+} affinity resin and the pET15b vector were purchased from Novagen. Recombinant FAS was a gift from H. Fu (Emory University) and J. Collier (Harvard Medical School).

Engineering N223-53. DNA encoding the carboxyl-terminal 223 amino acids of *exoT* (22) was amplified by the introduction of unique *Nde*I and *Bam*HI restriction sites at its 5′ and 3′ termini, respectively. The amplified DNA was digested with *Nde*I and *Bam*HI, and the product was ligated into the *Nde*I-*Bam*HI restriction sites of pET15b, yielding pET15bN223-53. The DNA encoding N223-53 was subjected to DNA sequencing to confirm that mutations were not generated during DNA amplification. N223-53 was composed of the 223 amino-terminal amino acids of Exo53 in frame with an amino-terminal 21-amino-acid sequence encoded by the vector.

* Corresponding author. Mailing address: Department of Microbiology, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226. Phone: (414) 257-8412. Fax: (414) 266-8522. E-mail: toxin@post.its.mcw.edu.

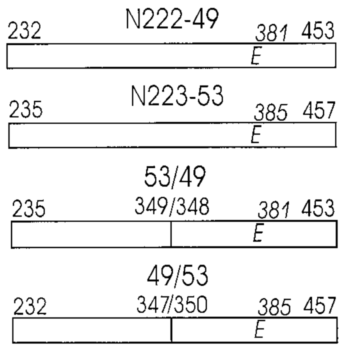

FIG. 1. Schematic representation of carboxyl-terminal deletion proteins of exoenzyme S. N222-49 represents a protein containing the carboxyl-terminal 222 amino acids of ExoS; N223-53 represents a protein containing the carboxyl-terminal 223 amino acids of Exo53. 53/49 and 49/53 represent hybrid proteins composed of the indicated regions of N222-49 and N223-53. Proteins were engineered as described in Materials and Methods. Residues are numbered with respect to the native proteins, ExoS and Exo53. E, glutamic acid.

Site-directed mutagenesis. Site-directed mutagenesis was performed on a single-stranded M13 DNA template by using a Sculpture Kit from Amersham. Introduction of a unique *Sma*I restriction site into the DNA encoding residues including and adjacent to position 347 of N222-49 was performed with the oligonucleotide 5′ GACACCGGGGTCCCGGGAGGTGG3′ (the nucleotide sequence represents the complement of the coding strand; boldfaced bases represent the mutation). Substitution of alanine for the glutamic acid at residue 385 (E385A) in N223-53 was performed with the oligonucleotide 5′ GTAGAG GATC**CG**CTGCTCATCG3′. Mutations were confirmed by sequencing phage single-stranded DNA. The entire open reading frame encoding N223-53 was sequenced to confirm that secondary mutations had not occurred.

Engineering 49-53 hybrid proteins. Two hybrid proteins composed of reciprocal halves of N222-49 and N223-53 were engineered. 49/53 encoded residues 232 to 347 of ExoS fused in frame to residues 350 to 457 of Exo53, and 53/49 encoded residues 235 to 349 of Exo53 fused in frame to residues 348 to 453 of ExoS (Fig. 1). DNAs encoding these hybrid proteins were generated by exchanging the respective DNA fragments of each gene at a unique *Sma*I restriction site at the junction point of the DNA encoding the hybrid proteins and a *Bam*HI restriction site which was located downstream of the translational termination codon of each gene. The *Sma*I restriction site occurred naturally within *exoT* and was engineered into *exoS* as described above. The resulting plasmids, termed pET49/53 and pET53/49, encoded the 49/53 and 53/49 proteins, respectively. 49/53 and 53/49 were expressed in *E. coli* and purified as described below for other His_6 fusion proteins.

Expression and purification of N223-53 and its derivatives. N223-53 and related proteins were expressed in *E. coli* BL21 (DE3) and purified by Ni^{2+} affinity chromatography followed by gel filtration (Sephacryl 200-HR) as previously described (17). Purified proteins were stored in 40% glycerol at −20°C. Immunoblotting with anti-ExoS immunoglobulin G was performed on the purified proteins as previously described (14). Immunoblots were probed with ^{125}I-protein A.

Determination of enzymatic activities. Linear velocities and kinetic constants for NAD and SBTI in the ADP-ribosyltransferase reaction were determined by measuring the incorporation of [^{32}P-adenylate phosphate] NAD into SBTI as previously described (17). Reaction mixtures contained (in 25 μl) 0.2 M sodium acetate (pH 6.0), 30 μM [^{32}P-adenylate phosphate] NAD (specific activity, 1 Ci/mmol), 30 μM SBTI, 0.25 μM recombinant FAS, and an aliquot of N222-49, N222-53, or a hybrid of the two which had been diluted into 25 mM Tris (pH 7.6) containing 0.1 mg of egg albumin/ml. Assays were performed at room temperature, and 25-μl aliquots were spotted onto trichloroacetic acid (TCA)-saturated Whatman 3MM chromatography paper at the following time points: 2.5, 5, and 10 min for N222-49 and 49/53 and 1, 2, and 3 h for N222-53 and 53/49. The sheets of chromatography paper were washed twice for 30 min with 7.5% TCA and once with methanol and then dried. Radioactivity was detected by scintillation counting.

(i) Kinetic analysis. (a) With different concentrations of 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 NAD was 30 μM, and that of SBTI was between 19.5 and 750 μM. Other components in the reaction mixture were as described for the velocity assay above. At the appropriate time, an aliquot (20 μl) was removed and spotted onto TCA-saturated paper. The amount of SBTI utilized was <10%. Data were transformed to the Lineweaver-Burk equation with the assistance of Enzfitter (Elsevier, Cambridge, United Kingdom).

(b) With different concentrations of NAD. The final concentration of SBTI was 30 μM, and that of NAD was between 19.5 and 333 μM. Assays were performed as described in the preceding section. The utilization of NAD was <10%.

```
                         A                        B
232 SADKALADGLVKRFGADAEKYLGRQPGGIHSDAEVMALGLYTGIHYADLN
    ..|||||||||.:|| :||.||| :|:| .||||||||||||. .|..||
235 PVDKALADGLVEHFGLEAEQYLGEHPDGPYSDAEVMALGLYTNGEYQHLN

282 RALRQGQELDAGQKLIDQGMSAAFEKSGQAEQVVKTFRGTRGGDAFNAVE
    |.||||.|||||| ||||||||||||||.|||||||||||.| |||:||.
285 RSLRQGRELDAGQALIDQGMSAAFEKSGPAEQVVKTFRGTQGRDAFEAVK
                                                 C
332 EGKVGHDDGYLSTSLNPGVARSF.GQGTISTVFGRSGIDVSGISNYKNEK
    ||.||||.|||||| :|:||||| |||||.|:|||||||||:||   :|.
335 EGQVGHDAGYLSTSRDPSVARSFAGQGTITTLFGRSGIDVSEISIEGDEQ

381 EILYNKETDMRVLLSASDEQGVTRRVLEEAALGEQSGHSQGLLDALDLAS
    ||||:|:|||||||||.|:|||||||||||.|||.|||::|||||||||.
385 EILYDKGTDMRVLLSAKDGQGVTRRVLEEATLGERSGHGEGLLDALDLAT
        D
431 KPERSGEVQEQDVRLRMRGLDLA
     .:|||..||||:||||||||||
435 GTDRSGKPQEQDLRLRMRGLDLA
```

FIG. 2. BESTFIT analysis of N222-49 and N223-53. Residues for N222-49 (upper sequence) and N223-53 (lower sequence) are numbered with respect to ExoS and Exo53, respectively. N222-49 and N223-53 showed 84% amino acid similarity (indicated by periods or colons) and 76% amino acid identity (indicated by vertical lines). Boldfaced sequences, designated A through D, represent four regions of N222-49 and N223-53 which show low-level primary amino acid homology. The boldfaced E381 in N222-49 and E385 in N223-53 represent candidate active site residues in the respective proteins.

(ii) NAD glycohydrolase reaction. Linear velocities for the NAD glycohydrolase reaction were determined as the release of [^{3}H]nicotinamide from [^{3}H-nictinamide]NAD as previously described (5). Briefly, reaction mixtures contained (in 25 μl) 0.2 M sodium acetate (pH 6.0), 30 μM [^{3}H-nicotinamide]NAD (specific activity, 1 Ci/mmol), an aliquot of N222-49 or N223-53, plus or minus 0.25 μM recombinant FAS. At the appropriate time, 10 μl of 1.0 M sodium borate (pH 8.0) was added to stop the reaction, and then the [^{3}H]nicotinamide was extracted by addition of 225 μl of H_2O-saturated ethyl acetate. The sample was centrifuged for 2 min at 13,800 × *g*, and radioactivity in the ethyl acetate phase was determined by liquid scintillation counting. Specific activities were determined in reactions in which <10% of the NAD had been utilized.

RESULTS

Expression of N223-53. Although Exo53 and ExoS possess 76% primary amino acid homology, Exo53 possesses only 0.2% of the ADP-ribosyltransferase activity of ExoS (22). Native and recombinant forms of Exo53 and ExoS exist as aggregates and are difficult to purify to homogeneity (14), which limits their use in biochemical studies. More amenable to biochemical analysis is a deletion protein of ExoS, termed N222-49, which is composed of its carboxyl-terminal 222 amino acids and which has been shown to possess FAS-dependent ADP-ribosyltransferase activity (12). BESTFIT alignment (Fig. 2) of N222-49 with the homologous region of Exo53 (represented by the 223 carboxyl-terminal residues of Exo53, termed N223-53) showed that N222-49 and N223-53 possessed 76% identity and 84% similarity at the primary amino acid level. This alignment shows that there is a high degree of homology between the two proteins, with only four regions possessing little primary amino acid homology. These four regions have been designated A through D. The biochemical and immunological analyses described in the present study will predict functions for several of these regions of limited homology.

Both N223-53 and N222-49 were expressed in *E. coli* as His_6 fusion proteins in stable and soluble forms under the regulation of the pET expression system. Following gel filtration chromatography, both N222-49 and N223-53 were purified to >80% homogeneity as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE analysis also showed that N223-53 and N222-49 migrated with apparent molecular masses of 30 and 27 kDa, respectively (Fig. 3). Immunoblot analysis showed that N223-53 reacted weakly with antiserum that was prepared against the 49-kDa form of exoenzyme S

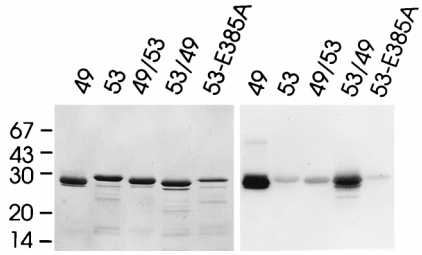

FIG. 3. Expression and immunoreactivity of carboxyl-terminal deletion proteins of ExoS and Exo53. The indicated proteins were purified and subjected, in duplicate, to SDS-PAGE in the presence of β-mercaptoethanol. The left panel is a photograph of the Coomassie blue-stained gel. The right panel is a photograph of an autoradiogram of an immunoblot that was probed with the anti-49-kDa form of exoenzyme S (ExoS) immunoglobulin G followed by ^{125}I-protein A. The positions of molecular size markers (in kilodaltons) are shown on the left.

(Fig. 3). This showed that while the 49- and 53-kDa forms of exoenzyme S share immunogenic epitopes, there appears to be a 49-kDa specific epitope within the carboxyl-terminal 222 amino acids of ExoS.

ADP-ribosyltransferase activity of N223-53. In a linear velocity reaction, N223-53 catalyzed the ADP-ribosylation of SBTI at 0.4% of the rate of catalysis by N222-49 (Table 1), with the expression of ADP-ribosyltransferase activity being FAS dependent. N223-53 catalyzed the ADP-ribosylation of the Ras protein at about 1% of the rate of catalysis by N222-49 (data not shown). The inability to purify sufficient amounts of recombinant Ras (11a) prevented its use for the determination of the kinetic properties of ExoS and Exo53. Kinetic analysis of the ADP-ribosylation of SBTI (Table 2) showed that N223-53 and N222-49 exhibited some changes in their affinities for binding NAD and SBTI. Relative to N222-49, N223-53 showed a threefold-lower $K_{m(app)}$ for NAD and a fivefold-greater $K_{m(app)}$ for SBTI. In contrast, N223-53 possessed a 60-fold reduction in the maximum velocity of the reaction when variable concentrations of SBTI were used and a 450-fold reduction in the maximum velocity of the reaction when various concentrations of NAD were used. This indicated that the primary defect of N223-53 in the ADP-ribosylation reaction was a reduction in the rate of catalysis. Titration of FAS in the ADP-ribosyltransferase reactions showed that N223-53 and N222-49 possessed identical activation curves, which indicated that two proteins have similar affinities for FAS (16a).

TABLE 1. Catalytic activities of N222-49 and N223-53

Activity	Enzyme	Sp act[a]	Relative activity[b]	FAS dependence[c]
ADP-ribosyltransferase	N222-49	68 ± 13	100	+
	N223-53	0.3 ± 0.1	0.4	+
	N223-53	0.3 ± 0.2	100	+
	N223-53E385A	Not detected	<1	
	N222-49	66.1 ± 3.5	100	ND[d]
	N223-53	Not detected	<1	ND
	53/49	0.4 ± 0.1	6	ND
	49/53	45.9 ± 8.4	70	ND
NAD glycohydrolase	N222-49	2.8 ± 0.2	100	+
	N223-53	0.06 ± 0.01	2	+

[a] Specific activity was expressed as picomoles of ADP-ribose incorporated into SBTI or moles of NAD hydrolyzed per minute per mole of enzyme.
[b] Activities were normalized to 100% of either N222-49 or N223-53 activity, as indicated for each set of experiments.
[c] +, expression of catalytic activity required the presence of FAS.
[d] ND, not determined.

TABLE 2. Kinetic constants for N222-49 and N223-53 in the ADP-ribosylation of SBTI[a]

Substrate varied	Enzyme	K_m (μM)	Velocity (V) (mol/min/mol of enzyme)	V/K_m
NAD	N222-49	40 ± 9	308 ± 45	7.7
	N223-53	15 ± 2	0.7 ± 0.4	0.04
SBTI	N222-49	49 ± 20	216 ± 63	4.4
	N223-53	266 ± 133	3.7 ± 0.1	0.01

[a] ADP-ribosylation of SBTI was assayed, with various amounts of NAD or SBTI (19.5 to 333 μM), as described in Materials and Methods.

NAD glycohydrolase activity of N223-53. The ability of N223-53 to catalyze the NAD glycohydrolase reaction was also determined. Under linear velocity conditions, N223-53 catalyzed the NAD glycohydrolase reaction at 0.06 mol of NAD hydrolyzed/min/mol of N223-53 while N222-49 catalyzed the NAD glycohydrolase reaction at 2.8 mol of NAD hydrolyzed/min/mol of N222-49 (Table 1). Both N223-53 and N222-49 required FAS for the expression of NAD glycohydrolase activity.

ADP-ribosyltransferase activity of the 53/49 and 49/53 hybrid proteins. To determine whether the catalytic defect of N223-53 was due to its overall amino acid composition or to specific regions within its primary amino acid sequence, two hybrid proteins of N222-49 and N223-53, termed 53/49 and 49/53, were engineered and subjected to analysis for ADP-ribosyltransferase activity. 53/49 and 49/53 consisted of reciprocal halves of the catalytic domains of ExoS and Exo53 (Fig. 1). Both 53/49 and 49/53 were expressed in *E. coli* as soluble, stable proteins which could be purified with yields and purities comparable to those of the parental proteins (Fig. 3). Linear velocity assays showed that 49/53 catalyzed the ADP-ribosylation reaction at 70% of the rate of N222-49 catalysis while 53/49 catalyzed the ADP-ribosylation reaction at 6% of the rate of N222-49 catalysis. This indicated that the primary catalytic defect of N223-53 resided between residues 235 and 349 of N223-53 and that the carboxyl-terminal region of N223-53 (amino acids 350 to 457) was essentially competent for catalysis.

Immunological reactivities of ExoS-related proteins. 53/49 and 49/53 were evaluated for their immunoreactivities to an anti-49-kDa form of exoenzyme S antiserum (14). Figure 3 demonstrates that antiserum specific for the 49-kDa form of exoenzyme S showed similar reactivities to N222-49 and the 53/49 protein while N223-53 and the 49/53 protein reacted weakly with the antiserum. These data indicate that the immunodominant epitope(s) specific for ExoS on the N222-49 and 53/49 proteins is localized to the carboxyl-terminal 106 amino acid residues.

E385 as an active site residue of N223-53. We have recently identified E381 as a candidate active site residue of ExoS (17). BESTFIT alignment (GCG, Inc., Madison, Wis.) of N222-49 and N223-53 predicted that E385 of Exo53 was the homolog of E381 of ExoS (Fig. 2). Utilizing site-directed mutagenesis, E385 of Exo53 was changed to an alanine, yielding N223-53E385A. N223-53E385A was expressed as a stable, soluble peptide in *E. coli* (Fig. 3). In a linear velocity assay, N223-53E385A failed to catalyze the ADP-ribosyltransferase reaction at detectable levels (Table 1). This was consistent with

E385A being the homolog of E381 of ExoS and a candidate active site glutamic acid of Exo53.

DISCUSSION

Since the discovery of exoenzyme S by Iglewski and coworkers (9), the molecular and biochemical relationships between the 53- and 49-kDa forms of this exoenzyme have been poorly defined. Recently, we have shown that the 53-kDa (Exo53) and 49-kDa (ExoS) forms of exoenzyme S are encoded by separate genes (16, 22). Although BESTFIT alignment of ExoS and Exo53 did not provide insight into the biochemical basis for the differential capacities of ExoS and Exo53 to catalyze the ADP-ribosyltransferase reaction (22), several mechanisms were proposed: (i) Exo53 possesses the potential for expression of ADP-ribosyltransferase activity at levels similar to those of ExoS, but expression is sterically blocked by its amino terminus; (ii) the low specific activity of Exo53 for ADP-ribosylation is due to a low affinity for either NAD or the target protein; and (iii) Exo53 lacks one or more amino acids required for expression of ADP-ribosyltransferase activity at levels observed for ExoS. The observation that N223-53 possesses only 0.4% of the ADP-ribosyltransferase activity of N222-49, a value that is similar to the relative activity of full-length Exo53 with respect to full-length ExoS (22), suggested that the low specific activity of Exo53 for ADP-ribosylation is intrinsic to the protein and not due to steric inhibition mediated by its amino terminus. The observation that N223-53 possesses similar binding affinities for NAD and SBTI, as observed for N222-49, suggested that a lower affinity for either substrate or target protein is not the primary reason for the low intrinsic ADP-ribosyltransferase activity of Exo53. The observed slow reaction velocity of N223-53 in the ADP-ribosylation reaction suggested that Exo53 possesses a defect in the ability to catalyze the ADP-ribosyltransferase reaction. This defect was apparent in both the NAD glycohydrolase and ADP-ribosyltransferase reactions, which suggested that the catalytic defect does not involve the direct transfer of ADP-ribose to an improperly aligned target protein but rather may involve a defect in the cleavage of NAD to ADP-ribose and nicotinamide. Analysis of the 49/53 and 53/49 proteins predicted that the catalytic defect is encoded between residues 235 and 349 of Exo53. Comparison of the BESTFIT alignment identified two regions of low-level homology between N222-49 and N223-53 which were designated A and B (Fig. 2). Either region may contain the defective region of Exo53. In contrast, it appears that the carboxyl-terminal half of N223-53, residues 350 to 457 of Exo53, is catalytically competent, since the 49/53 protein possesses about 70% of the ADP-ribosyltransferase activity of N222-49.

While members of the family of bacterial ADP-ribosylating exotoxins differ in their quaternary structures and primary amino acid sequences, these exotoxins possess conserved three-dimensional structures (2, 21) and conserved active site glutamic acid residues (1). Crystallographic studies have shown that the active sites of the heat-labile enterotoxin of *E. coli* (LT) and *P. aeruginosa* exotoxin A (ETA) (22) and those of diphtheria toxin and ETA (2) are essentially superimposable. The former alignment is especially noteworthy since LT and ETA share homology at only 3 of 43 amino acid residues within their active site regions, with one of the aligned amino acids being the active site glutamic acid. This glutamic acid has been shown to be involved in the catalytic portion of the ADP-ribosylation reaction (7, 18). Thus, although ExoS and Exo53 failed to show significant homology with other members of the family of bacterial ADP-ribosyltransferase exotoxins (16, 22), we propose that ExoS and Exo53 possess similar structure-function properties and a conserved three-dimensional structure within their active sites, which are common to the other members of the family of bacterial ADP-ribosylating exotoxins.

Extrapolation of the conservation of structure-function properties of other members of the family of bacterial ADP-ribosylating exotoxins (6) to ExoS and Exo53 leads to the prediction that both ExoS and Exo53 possess an active site glutamic acid. We have previously shown that E381 is a candidate active site residue of ExoS (16). The lack of detectable ADP-ribosyltransferase activity in N223-53E385A (Table 1) is consistent with the prediction that E385 of Exo53 is the homolog of E381 of ExoS and a candidate active site residue of Exo53. The bacterial ADP-ribosylating exotoxins can be aligned into subgroups according to the eukaryotic target protein that is ADP-ribosylated (13); members of each subgroup possess primary amino acid sequence homology immediately adjacent to the active site glutamic acid (17). Exo53 and ExoS appear to represent a unique subgroup based on the primary amino acid sequences adjacent to their respective active site glutamic acids. Placing ExoS and Exo53 into a unique subgroup within the family of bacterial ADP-ribosylating exotoxins was not predicted, since ExoS and Exo53 had been shown to share biochemical properties with other members of this family of exotoxins, including LT/cholera toxin (CT) and C3. However, in retrospect, ExoS and Exo53 possess quantitative catalytic properties that are different than those of LT/CT and C3. For example, although both LT/CT and ExoS and Exo53 require a eukaryotic accessory protein to express ADP-ribosyltransferase activity (11, 20), the eukaryotic accessory protein of LT and CT, ARF, enhances expression of ADP-ribosyltransferase activity while that of ExoS and Exo53, FAS, is absolutely required for expression of ADP-ribosyltransferase activity. While C3, ExoS, and Exo53 ADP-ribosylate small-molecular-weight GTP-binding proteins, the subsets of small-molecular-weight GTP-binding proteins that are the preferred targets for ADP-ribosylation differ (4, 10).

Earlier we reported the production of an antiserum to the 49-kDa form of exoenzyme S which was more reactive to the 49-kDa form of exoenzyme S (ExoS) than to the 53-kDa form (Exo53) (14). Figure 3 showed that this antiserum was more reactive to N222-49 and the 53/49 protein than to N223-53 and the 49/53 protein, which localized an ExoS-specific epitope to the carboxyl-terminal 107 amino acids of ExoS. BESTFIT alignment identified two regions of low-level amino acid homology between ExoS and Exo53 (Fig. 2), corresponding to residues 375 to 378 and 430 to 438 of ExoS, designated C and D, respectively (Fig. 2). While either region may encode an ExoS-specific epitope, assuming that the active site of ExoS aligns with other ADP-ribosyltransferases, we would predict that residues between positions 375 and 378 are located within the interior of the protein. Thus, we propose that residues between positions 430 and 438 of ExoS comprise an ExoS-specific epitope.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grants from the National Institutes of Health (RO1-AI-30162 and KO4-AI-01087 to J.T.B. and RO1-AI-31665 and KO4-AI-01289 to D.W.F.).

We thank Haian Fu and John Collier for FAS preparations.

REFERENCES

1. **Carroll, S. F., and R. J. Collier.** 1984. NAD binding site of diphtheria toxin: identification of a residue within the nicotinamide subsite by photochemical modification by NAD. Proc. Natl. Acad. Sci. USA **81:**3307–3311.
2. **Choe, S., M. J. Bennett, G. Fujii, P. M. G. Curmi, K. A. Kantardjieff, R. J. Collier, and D. Eisenberg.** 1992. The crystal structure of diphtheria toxin. Nature (London) **357:**216–222.

3. **Coburn, J., A. V. Kane, L. Feig, and D. M. Gill.** 1991. *Pseudomonas aeruginosa* exoenzyme S requires a eukaryotic protein for ADP-ribosyltransferase activity. J. Biol. Chem. **266:**6438–6446.
4. **Coburn, J., R. T. Wyatt, B. H. Iglewski, and D. M. Gill.** 1989. Several GTP-binding proteins, including $p21^{cHras}$, are preferred substrates of *Pseudomonas aeruginosa* exoenzyme S. J. Biol. Chem. **264:**9004–9008.
5. **Cortina, G., and J. T. Barbieri.** 1989. Role of tryptophan 26 in the NAD glycohydrolase reaction of the S1 subunit of pertussis toxin. J. Biol. Chem. **264:**17322–17328.
6. **Domenighini, M., and R. Rappuoli.** 1996. Three conserved consensus sequences identify the NAD-binding site of ADP-ribosylating enzymes, expressed by eukaryotes, bacteria, and T-even bacteriophages. Mol. Microbiol. **21:**667–674.
7. **Douglas, C. M., and R. J. Collier.** 1990. *Pseudomonas aeruginosa* exotoxin A: alterations of biological and biochemical properties resulting from mutation of glutamic acid 553 to aspartic acid. Biochemistry **29:**5043–5049.
8. **Fu, H., J. Coburn, and R. J. Collier.** 1993. The eukaryotic host factor that activates exoenzyme S of *Pseudomonas aeruginosa* is a member of the 14-3-3 protein family. Proc. Natl. Acad. Sci. USA **90:**2320–2324.
9. **Iglewski, B. H., J. Sadoff, M. J. Bjorn, and M. S. Maxwell.** 1978. *Pseudomonas aeruginosa* exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from toxin A. Proc. Natl. Acad. Sci. USA **75:**3211–3215.
10. **Jung, M., I. Just, J. van Damme, J. Vandekerckhove, and K. Aktories.** 1993. NAD-binding site of the C3-like ADP-ribosyltransferase from *Clostridium limosum*. J. Biol. Chem. **268:**23215–23218.
11. **Kahn, R. A., and A. G. Gillman.** 1986. The protein cofactor necessary for ADP-ribosylation of G_S by cholera toxin is itself a GTP-binding protein. J. Biol. Chem. **261:**7906–7911.
11a. **Knight, D. A., and J. T. Barbieri.** Unpublished data.
12. **Knight, D. A., V. Finck-Barbançon, S. M. Kulich, and J. T. Barbieri.** 1995. Functional domains of *Pseudomonas aeruginosa* exoenzyme S. Infect. Immun. **63:**3182–3186.
13. **Krueger, K. M., and J. T. Barbieri.** 1995. The family of bacterial ADP-ribosylating exotoxins. Clin. Microbiol. Rev. **8:**34–47.
14. **Kulich, S. M., D. W. Frank, and J. T. Barbieri.** 1993. Purification and characterization of exoenzyme S from *Pseudomonas aeruginosa* 388. Infect. Immun. **61:**307–313.
15. **Kulich, S. M., D. W. Frank, and J. T. Barbieri.** 1995. Expression of recombinant exoenzyme S of *Pseudomonas aeruginosa*. Infect. Immun. **63:**1–8.
16. **Kulich, S. M., T. Yahr, L. Mende-Mueller, J. T. Barbieri, and D. W. Frank.** 1994. Cloning the structural gene encoding exoenzyme S of *Pseudomonas aeruginosa*. J. Biol. Chem. **269:**10431–10437.
16a. **Liu, S., and J. T. Barbieri.** Unpublished data.
17. **Liu, S., S. M. Kulich, and J. T. Barbieri.** 1996. Identification of E381 as a candidate active site residue of *Pseudomonas aeruginosa* exoenzyme S. Biochemistry **35:**2754–2758.
18. **Lobet, Y., C. W. Cluff, and W. Cieplak, Jr.** 1991. Effect of site-directed mutagenic alterations on ADP-ribosyltransferase activity of the A subunit of *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **59:**2870–2879.
19. **Nicas, T. I., and B. H. Iglewski.** 1984. Isolation and characterization of transposon-induced mutants of *Pseudomonas aeruginosa* deficient in production of exoenzyme S. Infect. Immun. **45:**470–474.
20. **Noda, M., S.-C. Tsai, R. Adamik, J. Moss, and M. Vaughan.** 1990. Mechanism of cholera toxin activation by a guanine nucleotide-dependent 19 kDa protein. Biochim. Biophys. Acta **1034:**195–199.
21. **Sixma, T. K., S. E. Pronk, K. H. Kalk, E. S. Wartna, B. A. M. van Zanten, B. Witholt, and W. G. J. Hol.** 1991. Crystal structure of a cholera toxin-related heat-labile enterotoxin from *E. coli*. Nature (London) **351:**371–377.
22. **Yahr, T. L., J. T. Barbieri, and D. W. Frank.** 1996. Genetic relationship between the 53- and 49-kDa forms of exoenzyme S from *Pseudomonas aeruginosa*. J. Bacteriol. **178:**1412–1419.