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Functional Domains of *Pseudomonas aeruginosa* Exoenzyme S

DAVID A. KNIGHT, VIVIANE FINCK-BARBANÇON, SCOTT M. KULICH, AND JOSEPH T. BARBIERI*

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

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Recombinant exoenzyme S (rHisExoS) of *Pseudomonas aeruginosa* was expressed in *Escherichia coli* as a soluble, cytosolic His fusion protein. rHisExoS was purified by Ni^{2+}-affinity chromatography in the presence of protease inhibitors without detectable degradation. rHisExoS possessed a specific activity (within twofold) for the factor-activating exoenzyme S-dependent ADP-ribosylation of soybean trypsin inhibitor (SBTI) similar to that of native exoenzyme S. Analysis of several deletion peptides showed that ΔN222, which encoded the carboxyl-terminal 222 amino acids of exoenzyme S, possessed factor-activating exoenzyme S-dependent ADP-ribosyltransferase activity. ΔN222 catalyzed the ADP-ribosylation of SBTI at a rate sixfold greater than rHisExoS. Relative to rHisExoS, ΔN222 had a similar affinity for NAD, a threefold greater affinity for SBTI, and a four- to eightfold greater k_{cat} for the ADP-ribosylation of SBTI. Like native exoenzyme S, rHisExoS chromatographed as an aggregate with an apparent molecular mass of >300 kDa. In contrast, ΔN222 did not chromatograph as an aggregate, which showed that the amino-terminal 99 amino acids of exoenzyme S were responsible for the aggregation phenotype.

Pseudomonas aeruginosa produces two ADP-ribosyltransferases, exotoxin A and exoenzyme S (ExoS) (15). Exotoxin A and ExoS differ with respect to the eukaryotic proteins that are targeted for ADP-ribosylation (3, 4, 6) and by the requirement of ExoS for a eukaryotic accessory protein, termed factor-activating exoenzyme S (FAS), to express ADP-ribosyltransferase activity in vitro (5). Fu et al. (12) have cloned the gene encoding FAS from a bovine brain cDNA library and showed that FAS is a member of the 14-3-3 family of eukaryotic proteins. Although the protein that is targeted for ADP-ribosylation in vivo has not been defined, ExoS ADP-ribosylates a number of eukaryotic proteins in vitro, including the Ras protein and several other low-molecular-weight GTP-binding proteins, vimentin, and soybean trypsin inhibitor (SBTI) (6). SBTI is used as a convenient target to measure the in vitro ADP-ribosyltransferase activity of ExoS. ExoS has been implicated as a virulence determinant of *P. aeruginosa* in burn wounds and chronic lung infections (19).

ExoS is purified from the culture supernatant fluid of *P. aeruginosa* 388 as an aggregate composed of a 53-kDa protein and a 49-kDa protein. The 49-kDa protein of ExoS possesses enzymatic activity following elution from sodium dodecyl sulfate (SDS)-polyacrylamide gels (5, 19) and has been designated the enzymatically active form of ExoS. The 53-kDa protein of ExoS does not possess apparent ADP-ribosyltransferase activity in vitro (19). While the 53-kDa protein appears to be related to the 49-kDa form of ExoS on the basis of their immunological cross-reactivity (16, 19), possession of a similar amino-terminal amino acid sequence (2), and possession of common internal peptides (14), recent studies indicate that the 53- and 49-kDa proteins are encoded by separate genes (17).

The structural gene for the 49-kDa form of ExoS (*exoS*) was cloned (18) and shown to possess ADP-ribosyltransferase activity when expressed in *Escherichia coli* (17). Although ExoS lacked overall amino acid homology with other bacterial ADP-ribosylating exotoxins, three primary amino acid sequences that showed homology with primary amino acid sequences making up the active site of *E. coli* heat-labile enterotoxin (LT) were identified (18).

Although ExoS was expressed as an enzymatically active form in *E. coli*, the protein was partially degraded during purification by conventional chromatography (17). In this study, we report the expression and purification of a stable, soluble, recombinant form of ExoS in *E. coli* and define the kinetic parameters for the ADP-ribosylation of SBTI. Two functions were localized within the primary amino acid sequence of ExoS. The 222 carboxyl-terminal amino acids catalyze the FAS-dependent ADP-ribosylation reaction of SBTI, while the amino-terminal 99 amino acids confer the aggregation phenotype.

MATERIALS AND METHODS

Materials. Reagents were purchased from Sigma unless otherwise stated. [^{32}P-adenylate phosphate]NAD was purchased from Dupont-New England Nuclear. Ni^{2+}-affinity resin and pET vectors were purchased from Novagen. Recombinant FAS was a gift from H. Fu (Emory University) and J. Collier (Harvard Medical School). Native ExoS was purified from *P. aeruginosa* 388 as previously described (16). Bovine serum albumin was purchased from Pierce Biochemicals.

Construction of vectors for expression of recombinant His-tagged ExoS (rHisExoS) and deletion peptides. PCR amplification was used to engineer a unique *Nde*I restriction site within the DNA encoding the translational start site of the *exoS* structural gene, using pT7ExoS as template DNA (17). An *Nde*I-*Bam*HI restriction fragment which encoded the entire *exoS* structural gene was then cloned into the respective restriction sites of pET16b. Restriction endonuclease digestion followed by ligation was used to generate several internal deletions within the *exoS* structural gene. Internal deletions within *exoS* were initially engineered in pT7ExoS and then subcloned into the pET expression vector, since *Pst*I, *Eag*I, and *Eco*RV were unique pairs of restriction sites within *exoS* of pT7ExoS. Briefly, pT7ExoS (17) was digested with either *Pst*I, *Eag*I, or *Eco*RV and then religated to delete specific internal coding sequences within the *exoS* structural gene. These internal deletions did not disrupt the coding frame of downstream sequences of ExoS (Fig. 1). DNA fragments (*Nde*I-*Bam*HI) containing internal deletions within *exoS* were isolated and ligated into the respective restriction sites of pET16b, yielding pETExoS expression vectors.

DNA encoding amino acids 1 through 231 of ExoS was deleted by PCR amplification of pT7ExoS to introduce a unique *Nsi*I restriction site immediately 5′ to the *Eag*I restriction site at the DNA encoding residue 232 of the *exoS* structural gene and a *Bam*HI restriction site 3′ to the translational termination of the *exoS* structural gene. This DNA fragment was digested with *Nsi*I and *Bam*HI and ligated into the respective restriction sites of pT7ExoS which lacked the *exoS* structural gene. The *Nde*I-*Bam*HI restriction site of this construct was ligated into the respective restriction sites of the pET vector, yielding pETΔN222.

DNA encoding amino acids 342 through 453 was deleted from the *exoS*

* Corresponding author. Phone: (414) 456-8412. Fax: (414) 266-8522. Electronic mail address: toxin@post.its.mcw.edu.

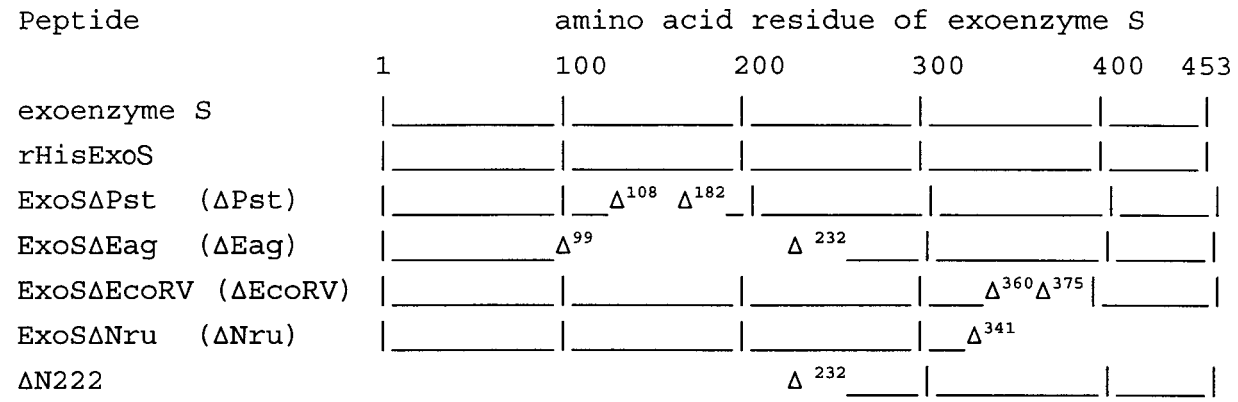

FIG. 1. Schematic representation of rHisExoS and its deletion peptides. rHisExoS and several of its deletion peptides are diagrammed. Superscript numbers represent the ExoS equivalent amino acid that is present at the junctions of deletion peptides.

structural gene by insertion of a transposon that contained a translational stop 3′ of residue 341 of the *exoS* structural gene. This vector, which possessed DNA encoding a peptide that contained the 341 amino-terminal residues of ExoS, was engineered by Dara Frank (Medical College of Wisconsin) and will be described in detail in a subsequent study (10a). The *Nsi*I-*Bam*HI DNA fragment encoding this deletion was ligated into the respective restriction sites of pT7 which lacked the *exoS* structural gene. The *Nde*I-*Bam*HI restriction site of this construct was ligated into the respective restriction sites of a pET vector, yielding pETExoSΔNru. A schematic representation of each ExoS deletion peptide is shown in Fig. 1.

Expression and purification of rHisExoS and ExoS deletion peptides. rHisExoS and ExoS deletion peptides were expressed in *E. coli* essentially as described by Novagen. Purification of nondegraded rHisExoS and ExoS deletion peptides required the addition of a mixture of protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, and leupeptin) to cell suspensions prior to their disruption with a French press and subsequent rapid isolation from cell extracts by Ni^{2+}-affinity chromatography.

A brief description of the purification protocol follows. Overnight cultures of *E. coli* BL21 carrying the appropriate pET vector were diluted 1/50 into L broth, either 400 or 800 ml, containing 100 μg of ampicillin per ml and shaken at 250 rpm and 30°C. After 2 h, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.6 mM and cultures were shaken for an additional 2 h. Cultures were centrifuged at 6,000 × *g* for 10 min, and the cell pellet was suspended in binding buffer (20 mM Tris-HCl [pH 7.9] containing 500 mM NaCl and 5 mM imidazole). Twelve micrograms of DNase I per ml, 12 μg of RNase A per ml, 24 μg of leupeptin per ml added in ethanol, 12 μg of aprotinin per ml added in H_2O, and 1.2 mM phenylmethylsulfonyl fluoride added in ethanol (final concentrations) were added, and cells were broken with a French press. Cell extracts were centrifuged at 30,000 × *g* for 8 min, and the soluble material was passed through a 0.45-μm-pore-size 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 100 mM imidazole. HisExoS proteins were eluted with binding buffer containing 0.5 M imidazole (2-ml fractions were collected).

ADP-ribosylation of SBTI: linear velocity. Reaction mixtures (50 μl) contained 0.2 M sodium acetate (pH 6.0), 30 μM [^{32}P-adenylate phosphate]NAD (specific activity, 1 Ci/mmol), 30 μM SBTI, 34.4 nM recombinant FAS, and an aliquot of rHisExoS or a deletion peptide of ExoS. rHisExoS or deletion peptides were diluted in 25 mM Tris (pH 7.6) containing 0.1 mg of egg albumin per ml. After incubation for 15 and 30 min at room temperature, an aliquot (20 μl) was removed and spotted onto trichloroacetic acid-saturated Whatman 3MM chromatography paper. Papers were washed for two 30-min periods with 100 ml of 7.5% trichloroacetic acid and once with methanol and dried. Radioactivity was detected by scintillation counting.

Kinetic analysis of ADP-ribosylation of SBTI by rHisExoS and ΔN222. (i) Variable SBTI. Reaction mixtures (25 μl) contained 0.2 M sodium acetate (pH 6.0), 30 μM [^{32}P-adenylate phosphate]NAD (specific activity, 1 Ci/mmol), a defined concentration of SBTI, 34.4 nM purified recombinant FAS, and an aliquot of rHisExoS or ΔN222. The final concentration of SBTI was varied between 2 and 50 μM by serial dilution in 10 mM Tris (pH 7.6) containing 0.1 mg of egg albumin per ml. After incubation for 20 min at room temperature, a 20-μl aliquot was removed and spotted onto trichloroacetic acid-saturated Whatman 3MM chromatography paper. Papers were washed for two 30-min periods with 100 ml of 7.5% trichloroacetic acid, soaked once in methanol, dried, and subjected to scintillation counting. The amount of SBTI utilized was less than 15%. Data were transformed to the Lineweaver-Burk equation with the assistance of Enzfitter (Elsevier, Cambridge, United Kingdom).

(ii) Variable NAD. Assays were performed as described above, with the following exceptions. The concentration of SBTI was 30 μM, while the concentration of NAD was varied between 8 and 200 μM (specific activity was adjusted by adding nonradiolabeled NAD to 0.75 μCi of [^{32}P-adenylate phosphate]NAD). After incubation for 20 min at room temperature, incorporation of [^{32}P-adenylate phosphate]NAD into trichloroacetic acid-precipitable material was determined as described above. In these experiments, <10% of the available NAD was utilized.

Gel filtration chromatography. rHisExoS and deletion peptides were subjected to Sephacryl 200HR gel filtration (10.5-ml column equilibrated in 25 mM Tris [pH 7.6] containing 50 mM NaCl). Column fractions (500 μl) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and then Coomassie staining. Proteins were quantitated with an AMBIS optical system.

Quantitation of peptides. The molar concentrations of rHisExoS and deletion peptides of rHisExoS were extrapolated by densitometry of peptide solutions that had been subjected to SDS-PAGE and stained with Coomassie blue, using an AMBIS optical imaging system. rHisExoS and deletion peptides of rHisExoS were normalized to a known concentration of bovine serum albumin with the assumption that these proteins possessed equimolar staining for Coomassie blue. The concentration of the SBTI stock was established at 1 mM by weight and assuming that 50% of the SBTI was available for ADP-ribosylation (15a).

RESULTS

Expression and purification of rHisExoS and deletion peptides. Previous studies showed that while recombinant ExoS catalyzed the FAS-dependent ADP-ribosylation of SBTI at a specific activity that was within twofold that of native ExoS, recombinant ExoS produced in *E. coli* was degraded during purification by conventional chromatographic techniques (17). Two modifications were introduced into the T7 expression system which allowed stable expression and purification of a soluble form of ExoS in *E. coli*. First, recombinant ExoS was expressed as a fusion protein in the pET expression system. This expression system introduced 21 amino acids at the amino terminus of ExoS which included 10 sequential His residues that were utilized for Ni^{2+}-affinity purification. Expression of rHisExoS and the deletion peptide ΔN222 is shown in Fig. 2. IPTG induced the expression of one unique peptide in *E. coli* strains carrying either pETHisExoS or pETΔN222 with apparent molecular masses of 52 and 26 kDa, respectively. The determined apparent molecular mass of 52 kDa for rHisExoS was consistent with the addition of the 21 amino acids encoded by the vector expression system of pET to ExoS. Immunoblot analysis of the cell lysates confirmed that both IPTG-induced proteins, rHisExoS and ΔN222, were recognized by anti-ExoS immunoglobulin G (IgG) (Fig. 2). Purification of nondegraded rHisExoS and rHisExoS deletion peptides required addition of a mixture of protease inhibitors immediately before cell disruption with a French press. The level of expression and final purity and yield of ΔN222 were greater than those achieved for

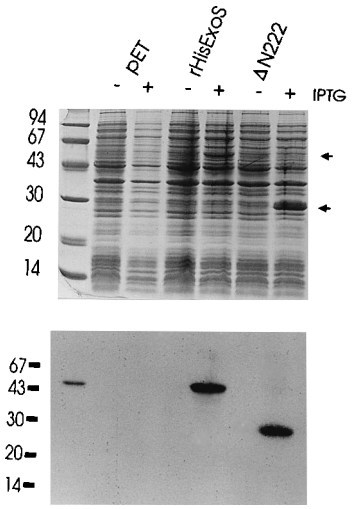

FIG. 2. Expression of rHisExoS and ΔN222 in *E. coli. E. coli* BL21 carrying pETrHisExoS or pETΔN222 was cultured in the absence (−) or presence (+) of IPTG as described in Materials and Methods. Cell extracts were subjected to SDS-PAGE, and the gels were stained with Coomassie blue (upper panel) or subjected to immunoblot analysis with α-ExoS IgG followed by detection with ^{125}I-protein A (lower panel). Molecular weight markers (10^3) were run in the left lane of both gels. The leftmost lane of the immunoblot includes an aliquot of native ExoS.

either rHisExoS or the other deletion peptides. The increased yield of ΔN222 was attributed to the fact that, unlike rHisExoS, ΔN222 was not expressed as a high-molecular-weight aggregate (data not shown).

Figure 1 shows a schematic of rHisExoS and the deletion peptides of ExoS that have been engineered. Following affinity purification, enriched preparations of rHisExoS and deletion peptides were obtained, except for ExoSΔEcoRV, which was not detected as a unique peptide either by Coomassie staining or on immunoblots that were probed with α-ExoS IgG (Fig. 3). Apparently ExoSΔEcoRV, which possessed an internal deletion of amino acids 360 to 375 of ExoS, was not stable in this expression system.

FAS-dependent ADP-ribosyltransferase activity of rHisExoS and deletion peptides. Under linear velocity conditions, rHisExoS ADP-ribosylated SBTI at a specific activity that was 70% that of native ExoS purified from *P. aeruginosa* 388

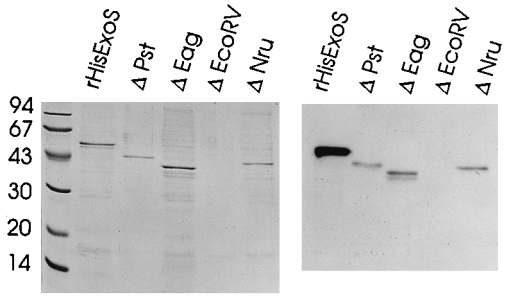

FIG. 3. Expression of rHisExoS and several deletion peptides in *E. coli*. rHisExoS and its deletion peptides were expressed in *E. coli* and purified from cell extracts as described in Materials and Methods and are represented in a schematic in Fig. 1. Purified proteins were subjected to SDS-PAGE. Gels were stained with Coomassie blue (left panel) or subjected to immunoblotting with α-ExoS IgG followed by ^{125}I-protein A (right panel). Migration of molecular weight marker proteins (10^3) are shown in the left lane of the Coomassie blue-stained gel.

TABLE 1. ADP-ribosylation of SBTI by rHisExoS and deletion peptides

Peptide	ADP-ribosyltransferase activity	
	% Sp act[a]	FAS dependence[b]
Native ExoS	100	+
rHisExoS	70	+
ExoSΔPst	67	+
ExoSΔEag	99	+
ExoSΔEcoRV	ND[c]	
ExoSΔNru	<1[d]	+
ΔN222	602	+

[a] Expressed relative to the ADP-ribosyltransferase activity of native ExoS, which is reported as 100%. Protein concentrations of the deletion peptides were estimated by densitometric scanning of proteins that had been subjected to SDS-PAGE followed by Coomassie staining relative to a known concentration of bovine serum albumin.

[b] +, no detectable ADP-ribosyltransferase activity was seen in the absence of FAS protein.

[c] ND, not detected; neither Coomassie blue-stained protein nor protein that reacted with α-ExoS IgG was detected at the expected molecular weight of ExoSΔEcoRV.

[d] No detectable activity at the limit of resolution of the assay, which was approximately 1%.

(Table 1). Like native ExoS, rHisExoS-catalyzed ADP-ribosylation of SBTI was dependent on the presence of FAS. These results indicated that rHisExoS possessed catalytic properties that were similar to those of native ExoS, which allowed the utilization of rHisExoS for further kinetic analysis.

The relative specific ADP-ribosyltransferase activities of a series of deletion peptides of ExoS with respect to that of native ExoS are shown in Table 1. Deletion of the 231 amino-terminal amino acids (ΔN222) or several internal sequences within the amino terminus of ExoS did not reduce the specific activity compared with that of native ExoS. Unexpectedly, ΔN222 catalyzed the ADP-ribosylation of SBTI at a sixfold greater rate than native ExoS. These deletion peptides of ExoS remained FAS dependent for the expression of ADP-ribosyltransferase activity. In contrast, rHisExoSΔNru, which lacked the 112 carboxyl-terminal amino acids of ExoS, did not possess detectable amounts of ADP-ribosyltransferase activity. Together, these data localized the FAS-dependent ADP-ribosyltransferase activity to the carboxyl-terminal 222 amino acids of ExoS.

FAS dependence for ADP-ribosylation of SBTI by ExoS. Utilizing the catalytically active recombinant deletion peptide of ExoS, ΔN222, and recombinant FAS, an evaluation of the absolute requirement for FAS in ExoS-catalyzed ADP-ribosylation of SBTI was determined in the absence of other *Pseudomonas* or eukaryotic factors. Neither ΔN222 at a concentration that ADP-ribosylated 10% of available SBTI in a 60-min assay nor a 10-fold greater concentration of ΔN222 yielded a detectable rate of ADP-ribosylation of SBTI in the absence of FAS (data not shown). The reciprocal experiment showed that, in the absence of ΔN222, 1× or 10× concentrations of FAS typically used in the ADP-ribosylation reaction did not catalyze detectable rates of ADP-ribosylation. Prolonged autoradiography of the gel containing these reaction mixtures failed to show detectable ADP-ribosylation of SBTI by ΔN222 or FAS in the absence of the reciprocal reagent. Rates of ADP-ribosylation were measured versus a control reaction mixture that did not contain either ΔN222 or FAS (data not shown).

Kinetic constants for FAS-dependent ADP-ribosylation of SBTI by rHisExoS and ΔN222. Analysis of the deletion peptides of ExoS localized the ADP-ribosyltransferase activity of ExoS to the carboxyl-terminal 222 amino acids and showed

TABLE 2. Kinetic constants of rHisExoS and ΔN222 in the ADP-ribosylation of SBTI

Peptide	K_{mapp} (μM)	k_{cat} [mol/(min/mol)]	k_{cat}/K_m
Variable NAD[a]			
rHisExoS	30 ± 10	25 ± 20	0.8
ΔN222	37 ± 0.7	190 ± 70	5.0
Variable SBTI[b]			
rHisExoS	8.0 ± 2.0	24 ± 10	3.0
ΔN222	3.1 ± 0.5	90 ± 20	30.0

[a] Constant SBTI, 30 μM; values are the average of two separate experiments ± standard deviation.
[b] Constant NAD, 30 μM; values are the average of four separate experiments ± standard deviation.

that ΔN222 possessed a ninefold greater rate of ADP-ribosylation than rHisExoS (Table 1). To resolve the mechanism for the increased rate of ADP-ribosylation catalyzed by ΔN222, kinetic parameters for the ADP-ribosylation of SBTI by rHisExoS and ΔN222 were determined (Table 2). Initial linear velocities for the ADP-ribosyltransferase reaction were proportional to the concentration of NAD or SBTI and achieved zero-order rates at higher NAD or SBTI concentrations. Lineweaver-Burk plots of the initial linear velocities as a function of NAD and SBTI followed Michaelis-Menten kinetics, which allowed the measurement of binding affinity for NAD and SBTI as well as k_{cat} determinations. At variable NAD concentrations, rHisExoS and ΔN222 possessed similar affinities for NAD, with K_ms of 30 and 37 μM, respectively. ΔN222 possessed an eightfold greater k_{cat} (190 [min^{-1}]) than rHisExoS (25 [min^{-1}]). The determined catalytic efficiencies (k_{cat}/K_m) were 5 for ΔN222 and 0.8 for rHisExoS. At variable SBTI concentrations, ΔN222 showed a threefold greater affinity for SBTI than rHisExoS, with K_ms of 3 and 8 μM, respectively. ΔN222 possessed a fourfold greater k_{cat} (90 [min^{-1}]) than rHisExoS (24 [min^{-1}]). At variable SBTI concentrations, the k_{cat}/K_m of ΔN222 was 10-fold greater than that of rHisExoS. These data establish the first determinations of the kinetic constants for ExoS in the ADP-ribosylation reaction and show that the primary difference between ΔN222 and rHisExoS in the ADP-ribosylation of SBTI is that ΔN222 possesses a greater k_{cat}.

Aggregation properties of rHisExoS and ExoS deletion peptides. Among the members of the family of bacterial ADP-ribosylating exotoxins, ExoS is unique with respect to its physical organization as a high-molecular-weight aggregate (16). Earlier studies showed that the native ExoS aggregate copurified with two proteins of subunit molecular masses of 53 and 49 kDa (19) and that urea and Triton X-100 stimulated the dissociation of the aggregate (16), which suggested that the ExoS aggregate was composed of both lipid and protein interactions. Like native ExoS, rHisExoS eluted in the void volume when subjected to gel filtration chromatography with Sephacryl S300HR under nondenaturing conditions (data not shown). Subsequent gel filtration analysis was performed with Sephacryl S200HR to generate a more accurate determination of the molecular mass of ΔN222 (Fig. 4). Both rHisExoS and the deletion peptide ExoSΔEag eluted in the void volume upon chromatography in Sephacryl S200HR. ExoSΔEag lacks the internal 100 to 232 amino acids of ExoS. This indicated that the aggregation phenotype did not reside within the area between residues 100 and 232 of ExoS. In contrast, ΔN222 did not elute in the void volume but eluted with an apparent molecular mass of approximately 64 kDa. This showed that the 99 amino-terminal residues of ExoS were responsible for the aggregation properties of ExoS and that ΔN222 migrated, in a native gel filtration system, as an apparent dimer with respect to its subunit molecular mass of 26 kDa.

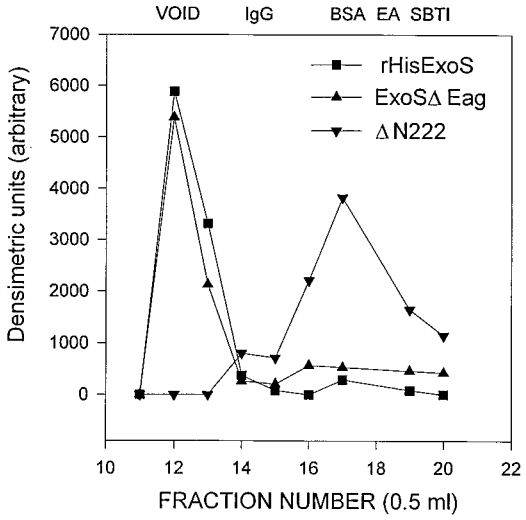

FIG. 4. Gel filtration chromatography of rHisExoS and several deletion peptides. rHisExoS (■), ExoSΔEag (▲), and ΔN222 (▼) were subjected to Sephacryl S200HR gel filtration chromatography (10.5 ml of resin equilibrated in 25 mM Tris-HCl [pH 7.6] containing 50 mM NaCl). Column fractions (0.5 ml) were collected, and an aliquot of each fraction was subjected to SDS-PAGE. The gel was stained with Coomassie blue, and proteins were quantitated by AMBIS optical densitometry and reported in arbitrary units. BSA, bovine serum albumin.

DISCUSSION

In this study, we report the expression and purification of a stable recombinant form of ExoS and of several deletion peptide derivatives of ExoS. rHisExoS possessed catalytic and physical properties that were similar to those of native ExoS. In addition, a functional map of ExoS which localized regions responsible for aggregation, activation by FAS, and ADP-ribosyltransferase activity was determined.

The organization of ExoS as a high-molecular-weight, noncovalently associated aggregate appears to be a property of the protein itself and does not require the presence of other *Pseudomonas* proteins, since rHisExoS and other recombinant forms of ExoS (Fig. 4) chromatograph as aggregates. Analysis of deletion peptides of ExoS showed that the 99 amino-terminal residues of ExoS were required for the expression of this aggregation phenotype. The fact that ΔN222 eluted with an apparent molecular mass of a dimer either may reflect the fact that ΔN222 is a dimer or may indicate the presence of noncovalently associated material bound to the protein. This anomalous chromatographic property may be relevant to the determination of the mechanism by which FAS interacts with ExoS, since in an analogous system phospholipids have been shown to modify the interactions between ADP-ribosylation factor (ARF) and cholera toxin (CT) (1, 23). Future studies will resolve the mechanism responsible for the aggregation of ExoS and determine the reason for the greater than subunit molecular mass of ΔN222.

ExoS possessed a K_m of 30 μM for NAD, similar to the affinity of NAD reported for diphtheria toxin (7), exotoxin A (10), and pertussis toxin (8) and greater than the affinity of CT and LT (22) for NAD. Like pertussis toxin, which possesses a micromolar binding affinity for the heterotrimeric G protein

transducin (9), ExoS possessed a micromolar K_m for SBTI, an in vitro eukaryotic target protein for ADP-ribosylation. Secondary plots of the initial linear velocities as a function of NAD and SBTI for the ADP-ribosylation of SBTI by ExoS fit the Lineweaver-Burk equation. This suggested that although ExoS possessed an absolute requirement for FAS for the expression of enzymatic activity, the binding of substrate (NAD) and target protein (SBTI) appeared to occur via a simple, noncooperative mechanism.

FAS-dependent ADP-ribosyltransferase activity was localized to the carboxyl-terminal 222 amino acids of ExoS. This was consistent with earlier studies that aligned three noncontiguous regions of the active site of LT and CT with three sequences within the carboxyl terminus of ExoS (18). The fact that ΔN222 retained the requirement of FAS for the expression of ADP-ribosyltransferase activity localized the FAS binding domain to the 222 carboxyl-terminal residues of ExoS. Indeed, the FAS dependency of ExoS for the expression of ADP-ribosyltransferase activity appears absolute, within the resolution of the assay. The absolute dependence of ExoS on the FAS protein to stimulate ADP-ribosylation resembles that observed for the stimulation of CT by ARF. Moss and coworkers (1) showed that in the absence of lipids and ARF CT catalyzed negligible levels of ADP-ribosyltransferase activity. However, the fact that ARF may cycle between the soluble and membrane components of the cell, along with the determination that ARF stimulation of CT involves both phospholipids and nucleotide components, may reflect a complex interaction between ARF and CT (1, 22, 23). Comparative analysis of the ARF-CT and FAS-ExoS interactions will facilitate advances in defining how these eukaryotic proteins regulate ADP-ribosyltransferase activity. Our current understanding of FAS-ExoS interactions does not resolve whether FAS activates the intrinsic enzymatic activity of ExoS or whether FAS plays a direct role in the ADP-ribosylation reaction.

The primary difference between the kinetic properties of ΔN222 and rHisExoS was that ΔN222 expressed a greater k_{cat} for the ADP-ribosylation of SBTI. With respect to rHisExoS, ΔN222 possessed a similar affinity for NAD and a threefold greater affinity for SBTI. The four- to eightfold increase in k_{cat} may reflect a more optimal alignment of residues that participate in the ADP-ribosylation reaction or may reflect the fact that only a portion of the molecules within the ExoS aggregate possesses ADP-ribosyltransferase activity.

The fact that ΔN222 possessed binding affinities for NAD and SBTI similar to those of rHisExoS and did not exist as an aggregate suggests that ΔN222 will be a useful target to study the ADP-ribosyltransferase activity of ExoS. Utilizing nomenclature that describes the "A:B" structure-function relationship among other bacterial ADP-ribosylating exotoxins (13), ΔN222 appears to represent the catalytic, or "A," domain of ExoS. Neither the binding, or "B," domain of ExoS nor the physiological processes involved in the entry of ExoS into eukaryotic cells have been defined. In fact, characterization of ExoS as an exotoxin awaits the identification of its cytotoxicity to cells in either culture or an animal model (2). Earlier studies reported that a transposon-generated mutant, *P. aeruginosa* 388 *exs*1::Tn*1*, which did not express ExoS was less virulent than parental strains (20, 21). Subsequent studies showed that the transposition within *P. aeruginosa* 388 *exs*1::Tn*1* had disrupted the function of the "*trans*-regulatory operon" which controls the expression of several *Pseudomonas* proteins, including ExoS. Down-regulation of any of these proteins may have contributed to the lower virulence of *P. aeruginosa* 388 *exs*1::Tn*1* (11). Future studies will be directed towards the generation of noncatalytic forms of ExoS that can be used to evaluate the role of ExoS in the pathogenesis of *P. aeruginosa*.

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