Expression and Secretion of the S-1 Subunit and C180 Peptide of Pertussis Toxin in *Escherichia coli*

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The structural gene of the S-1 subunit of pertussis toxin (rS-1) and the catalytic C180 peptide of the S-1 subunit (C180 peptide) were independently subcloned downstream of the *tac* promoter in *Escherichia coli*. Both constructions included DNA encoding for the predicted leader sequence of the S-1 subunit which was inserted between the *tac* promoter and the structural gene. *E. coli* containing the plasmids encoding for rS-1 and C180 peptide produced a peptide that reacted with anti-pertussis toxin antibody and had a molecular weight corresponding to that of the cloned gene; some degradation of rS-1 was observed. Extracts of *E. coli* containing plasmids encoding for rS-1 and the C180 peptide possessed ADP-ribosyltransferase activity. Subcellular fractionation showed that both rS-1 and the C180 peptide were present in the periplasm, indicating that *E. coli* recognized the pertussis toxin peptide leader sequence. The protein sequence of the amino terminus of the C180 peptide was identical to that of authentic S-1 subunit produced by *Bordetella pertussis*, which showed that *E. coli* leader peptidase correctly processed the pertussis toxin peptide leader sequence. Two single amino acid substitutions at residue 26 (C180I-26) and residue 139 (C180S-139) which were previously shown to reduce ADP-ribosyltransferase activity were introduced into the C180 peptide. C180I-26 possessed approximately 1% of the NAD-glycohydrolase activity of the C180 peptide, suggesting that tryptophan 26 functions in the interaction of NAD with the C180 peptide. In contrast, C180S-139 possessed essentially the same level of NAD-glycohydrolase activity as the C180 peptide, suggesting that glutamic acid 139 does not function in the interaction of NAD but plays a role in a later step in the ADP-ribosyltransferase reaction.

Bordetella pertussis, the etiologic agent of whooping cough, produces several factors which contribute to its pathogenesis (26). Two of these factors, pertussis toxin (PT) and filamentous hemagglutinin, also act as immunogens which stimulate production of protective antibodies against the bacterium (22). Present investigations have focused on the development of an acellular vaccine to replace the whole cell vaccine currently administered in the United States. One approach to the development of an acellular vaccine would be to use a nontoxic form of PT and filamentous hemagglutinin, alone or in concert, as the immunogen. The cloning of the PT operon in *Escherichia coli* (14, 18) has allowed the use of recombinant DNA techniques to attempt to modify PT such that the protein is nontoxic but retains immunogenic properties, allowing its use as an immunogen.

PT (molecular weight, 105,060 [18]) is composed of six noncovalently bound subunits designated S-1 through S-5 (24). There is one copy of each subunit, except for S-4, which is present in two copies. The S-1 subunit contains 235 amino acids (18) and catalyzes the covalent transfer of the ADP-ribose portion of NAD to G_i protein, the negative regulatory component of the adenylate cyclase complex (12). The remaining five subunits (S-2 through S-5) compose a pentamer which is responsible for delivering the S-1 subunit to the G_i protein.

We and others (1, 3, 5, 6, 20) have begun to define the functional residues of the S-1 subunit of PT with the goal of generating noncatalytic but immunologically conserved forms of the subunit amenable for administration as an acellular vaccine. The subunits of PT have been expressed in *E. coli* (2, 13, 17) and *Bacillus subtilis* (21) under the regulation of host bacterial promoters. Deletion mapping

showed that residues 2 through 180 possessed ADP-ribosyltransferase activity (1, 13, 20). Several amino acids within the S-1 subunit have been identified which are required for expression of catalytic activity (1, 6, 20). Burnette et al. (7) have also recently described the expression of nonfusion forms of the S-1 subunit. These investigators reported that the recombinant proteins were produced in large amounts and were localized within inclusion bodies of *E. coli*. They also observed that the leader sequence was processed with varying efficiencies.

In this study, we report the expression of a nonfusion form of the S-1 subunit (rS-1) and a catalytic peptide of the S-1 subunit which is composed of the first 180 amino-terminal residues (the C180 peptide). The peptides were expressed under the regulation of the *tac* promoter in *E. coli*. The recombinant peptides were soluble, secreted into the periplasm, and had their leader sequence correctly processed. Characterization of two mutants of the S-1 subunit indicated that tryptophan 26 functions in the binding and/or hydrolysis of NAD, while glutamic acid 139 plays a role in a later step in the ADP-ribosyltransferase reaction.

MATERIALS AND METHODS

Materials. Iodine-125 and [4-³H]NAD were purchased from Amersham Corp. (Arlington Heights, Ill.), and ³²P-NAD was purchased from Dupont, NEN Research Products (Boston, Mass.). Enzymes used in the manipulation of DNA were purchased from Molecular Biology Resources, Inc. (Milwaukee, Wis.) or New England BioLabs, Inc. (Beverly, Mass.). Prestained molecular weight markers used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Diversified Biotech, (Newton Centre, Mass.). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Research Organics, Inc. (Cleveland,

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FIG. 1. Protocol for construction of ptacS-1c and ptacC180. Symbols in the DNA fragment insert: □, *tac* promoter; ▨, leader sequence of the S-1 gene; ■, structural S-1 gene; □, terminator cassette.

Ohio). Protein determinations were made with the Protein Assay Reagent from Pierce Chemical Co. (Rockford, Ill.), with bovine serum albumin as a standard.

Bacterial strains and plasmids. pCDptac2 was obtained from C. M. Douglas and R. J. Collier (Harvard Medical School), and pUC18 was purchased from Pharmacia, Inc. (Piscataway, N.J.). Plasmids were maintained in *E. coli* JM103. Plasmid manipulations were performed essentially as described by Maniatis et al. (15).

Construction of ptacS-1c and ptacC180. rS-1 and C180 peptide were expressed under the regulation of the *tac* promoter in *E. coli* JM103. To facilitate insertion of DNA encoding for rS-1, the *tac* expression vector (pCDptac2) (10) was subjected to two modifications. The pUC8 polylinker in pCDptac2 was replaced with the analogous polylinker region of pUC18; this introduced several additional unique restric-

tion sites into the expression vector. Next, a segment of the expression vector was deleted at the *NaeI* and *PvuII* recognition sites. This removed DNA encoding for a unique *AvaI* restriction site in the expression vector which allowed a more convenient insertion of the S-1 gene. This modified plasmid was called ptac18 (Fig. 1).

DNA encoding for the S-1 subunit was inserted into ptac18 in two steps (Fig. 1). (i) A synthetic double-stranded oligonucleotide (shown below)

was inserted into the *BamHI* and *SalI* restriction sites of ptac18, yielding ptacCass. This synthetic oligonucleotide

inserted a 3-nucleotide spacer, 5'-GTT-3', between the *Bam*HI site of the *tac* promoter and the ATG (underlined) which signaled the start of translation of the S-1 gene. This nucleotide spacing has been used to express diphtheria toxin from pCDptac2 (R. J. Collier and co-workers, manuscript in preparation). The synthetic double-stranded oligonucleotide also encoded the 5' terminus of the S-1 gene through an *Ava*I restriction site, the first unique restriction site of the S-1 gene. (ii) DNA encoding for the remaining complementary sequence of the S-1 gene was inserted into ptacCass as an *Ava*I-*Hind*III DNA fragment. This *Ava*I-*Hind*III DNA fragment (isolated from pPT101, which contained the entire PT operon and was obtained from R. Rappuoli) contained DNA encoding for the remainder of the S-1 leader sequence, the entire coding region of the S-1 gene, and a terminator cassette. Insertion of the terminator cassette distal to the S-1 structural gene allowed convenient construction of a vector expressing the C180 peptide (1). The rS-1 expression vector was called ptacS-1c.

ptacS-1c was digested with *Nru*I and *Eco*RV, ligated, and transformed into *E. coli* JM103. Removal of DNA between the *Nru*I and the *Eco*RV sites produced a deletion within the S-1 structural gene, yielding a gene product comprising the S-1 leader sequence and amino acids 1 through 180 of the structural S-1 gene, followed by an isoleucine which was encoded by the terminator cassette. This construction was called ptacC180.

Subcellular fractionation of the C180 peptide and S-1 subunit. Overnight cultures of *E. coli* containing ptac18, ptacC180, or ptacS-1c were diluted into M-9 medium (15) supplemented with 0.3% glycerol, 0.3% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.005% thiamine hydrochloride, and 100 µg of ampicillin per ml and were incubated at 30°C with shaking at 250 rpm. When the cell suspension reached an optical density at 595 nm of 1, 1 mM IPTG was added and the incubation continued for 2 h. Extraction of the periplasm was performed as described by Ito et al. (11). The IPTG-induced culture (30 ml) was concentrated by centrifugation at $5,000 \times g$ for 6 min and suspended in 0.5 ml of 33 mM Tris hydrochloride (pH 8.0)–30% sucrose–2 µg of phenylmethylsulfonyl fluoride per ml. Lysozyme (150 µg in 25 µl of 0.1 M EDTA) was added to the cell suspension. After a 30-min incubation on ice, 25 µl of 1.0 M $MgCl_2$ was added and the cells were concentrated by centrifugation at $5,000 \times g$ for 10 min. The soluble material (periplasmic fraction) and the particulate material (cellular fractions) were assayed for alkaline phosphatase (periplasmic marker), for glucose-6-phosphate dehydrogenase (cellular marker) as recommended by the *Worthington Enzyme Manual* (L. A. Decker, Worthington Biochemical Corp., Freehold, N.J., 1977), and for the presence of the C180 peptide or rS-1 by immunoblotting.

Isolation of the C180 peptide. Isolation of the C180 peptide was performed as follows. A 7.0-liter culture of *E. coli* JM103(ptacC180) was grown in L broth (15) with constant O_2 sparging at 2 liters/min at 30°C. When the cells reached an optical density at 595 nm of 1, 1.0 mM IPTG was added and the incubation was continued for 4 h. Cells were then concentrated by centrifugation at $1,200 \times g$ for 25 min in an IEC PR6000 centrifuge and suspended in 140 ml of 33 mM Tris hydrochloride (pH 8.0)–30% sucrose–2 µg of phenylmethylsulfonyl fluoride per ml. Lysozyme (108 mg in 11.3 ml of 0.1 M EDTA) was added to the cell suspension. After a 30-min incubation on ice, 11.3 ml of 1.0 M $MgCl_2$ was added and the cells were concentrated by centrifugation at $5,000 \times g$ for 25 min. β -Mercaptoethanol (10 mM) was added to the

soluble material (periplasmic fraction). The periplasmic fraction (175 ml) was subjected to gel exclusion chromatography with Sephacryl S-200HR (Pharmacia). The S-200HR column (1,500-ml bed volume) was equilibrated in 25.0 mM Tris hydrochloride (pH 7.6) containing 10.0 mM β -mercaptoethanol. The flow rate was 90 ml/h, and the effluent was collected in 8-ml fractions. Fractions that contained the C180 peptide, identified by immunoblotting, were combined and labeled the S-200HR pool. The S-200HR pool was applied to a DEAE-Sephacel (Pharmacia) column (25-ml bed volume) which had been equilibrated in 25 mM Tris hydrochloride (pH 7.6) containing 10 mM NaCl. After the S-200HR pool (160 ml) was loaded, the column was washed with 4 volumes of equilibration buffer and 4 volumes of 25 mM Tris hydrochloride (pH 7.6) containing 50 mM NaCl. The C180 peptide was then eluted with 25 mM Tris hydrochloride (pH 7.6) containing 100 mM NaCl and labeled the DEAE pool. The DEAE pool was precipitated in 50% saturated ammonium sulfate. The precipitate was equilibrated in 25 mM Tris hydrochloride (pH 7.6) and labeled the C180 peptide.

Properties of the C180 peptide. (i) **N-terminal sequence.** The C180 peptide was subjected to SDS-PAGE, and proteins in the gel matrix were transblotted (16) to polyvinylidene difluoride membranes. Staining with amido black showed that a single predominant protein with an apparent molecular size of 21,000 daltons had been transferred to the membrane. Membrane pieces containing the 21,000-dalton band were inserted into the sequencing chamber of a pulsed-liquid-phase protein sequencer (model 477A; Applied Biosystems). The sequence of the first 14 amino acids from the N terminus of the 21,000-dalton band is presented in the Results section.

(ii) **Amino acid composition.** The amino acid composition of the C180 peptide was determined by using an amino acid analyzer (model 6300; Beckman Instruments, Inc., Fullerton, Calif.) after peptide hydrolysis in 6 N HCl at 110°C for 20 h.

(iii) **UV spectrum.** UV properties of the C180 peptide were determined by using a Beckman model DU-64 spectrophotometer and quartz cuvettes with a 1-cm light path.

Measurement of NAD-glycohydrolase activity. NAD-glycohydrolase activity was measured as the release of [3H]nicotinamide from [3H]NAD (9). A typical reaction mixture contained (in 25 µl) 0.1 M Tris hydrochloride (pH 8.0), 20.0 mM dithiothreitol, 1.0 mg of egg albumin per ml, [4-^3H]NAD (0.75 µCi; 2.2 Ci/mmol), and sample. The reaction mixtures were incubated for the indicated times at 37°C, and the reactions were stopped by the addition of 10 µl of 1.0 M boric acid (sodium salts, pH 9.0). Water-saturated ethyl acetate (250 µl) was added to each reaction mixture, which was then vortexed and centrifuged for 2 min at $13,800 \times g$ at room temperature. The ethyl acetate phase (100 µl) was added to 2 ml of Aqueous Counting Scintillant (Amersham Corp.), and radioactivity was determined by liquid scintillation counting. When measuring the effect of immunoglobulin G (IgG) on this reaction, we incubated peptides with IgG for 30 min at room temperature prior to the determination of enzyme activity. Total protein in the preincubation reaction mixtures was adjusted to 40 µg with the addition of the appropriate amount of egg albumin. This maintained the stability of the diluted C180 peptide.

ADP-ribosylation of G_i protein. Reaction mixture (100 µl) contained 0.1 M Tris hydrochloride (pH 8.0), 20.0 mM dithiothreitol, 2.0 mM ATP, human erythrocyte membranes (approximately 10 µg of protein), ^{32}P -NAD (1.0 µCi; 1,000 Ci/mmol), and sample. Samples were added last to start the reaction. After 1 h at 37°C, erythrocyte membranes were

concentrated by centrifugation at $13,800 \times g$ for 15 min, suspended in SDS-PAGE sample buffer containing β -mercaptoethanol, and subjected to 12.5% SDS-PAGE. ADP-ribosylated G_i protein was identified in the gel matrix by autoradiography. Exposure times were adjusted to allow the determination of a range of activities in constant proportion to the densitometric signal.

Preparation of anti-C180 peptide IgG. New Zealand White rabbits were prebled and subjected to the following immunization protocol. At 1-month intervals, rabbits were immunized intramuscularly and subcutaneously with 100 μ g of the C180 peptide in Freund complete adjuvant, then in Freund incomplete adjuvant, and subsequently in saline. At 1 to 2 weeks after immunization, rabbits were bled. The IgG fraction from preimmunized and immunized sera was obtained by protein A affinity chromatography, using the protocol described by the manufacturer (Pierce Chemical Co.). IgG was quantified by spectral determination, using a coefficient of 14 units of optical density at 280 nm for a 1% solution of IgG.

Immunoblotting. Cell extracts were subjected to 13.5% SDS-PAGE in the presence of β -mercaptoethanol. Proteins in the gel matrix were transferred electrophoretically to nitrocellulose and then probed with 125 I-labeled anti-PT antibody (the gift of J. L. Cowell and C. R. Manclark, Bureau of Biologics, Food and Drug Administration, Bethesda, Md.). Nitrocellulose was then washed, and immunoreactive proteins were identified by autoradiography. Exposure times were adjusted to allow the determination of a range of immunoreactive material in constant proportion to the densitometric signal.

RESULTS

Plasmid constructions. DNA encoding rS-1 and the C180 peptide was expressed under the regulation of the *tac* promoter in *E. coli* (Fig. 1). The 34-amino-acid leader sequence of the S-1 subunit (3, 4) was inserted upstream of the S-1 structural gene (235 amino acids). The predicted molecular size of the rS-1 with an uncleaved signal sequence was 29,978 daltons and that for the mature rS-1 was 26,225 daltons. The structural gene of the C180 peptide contained the 34-amino-acid leader sequence of the S-1 subunit and amino acids 1 through 180 of the S-1 subunit, followed by an isoleucine encoded by the expression vector. The predicted molecular size of the C180 peptide with uncleaved signal sequence was 24,163 daltons and that for the mature C180 peptide was 20,410 daltons (181 amino acids). Molecular sizes were calculated by using the PEP program of Bionet.

Expression of S-1 subunit and C180 peptide. IPTG-induced *E. coli* JM103(ptacC180) expressed a single peptide which reacted with anti-PT antibody (Fig. 2). The apparent molecular size of the immunoreactive peptide was 21,000 daltons; this peptide was designated the C180 peptide. IPTG-induced *E. coli* JM103(ptacS-1c) expressed several peptides which reacted with anti-PT antibody (Fig. 2). The largest immunoreactive peptide migrated with a relative mobility identical to that of the authentic S-1 subunit of PT and had an apparent molecular size of 30,000 daltons; this peptide was designated rS-1. IPTG-induced *E. coli* JM103(ptacS-1c) expressed several smaller immunoreactive peptides which reacted with anti-PT antibody. The predominant three immunoreactive peptides had apparent molecular sizes of 26,000, 24,000, and 23,000 daltons. These peptides were probably the products of protease digestion of rS-1. IPTG-induced *E. coli*(ptac18) did not express immunoreactive peptides (Fig. 2).

FIG. 2. Expression of the S-1 subunit and C180 peptide by *E. coli* JM103. Induction of the *tac* promoter by IPTG. *E. coli* JM103(ptac18), JM103(ptacC180), or JM103(ptacS-1c) was grown for 2 h at 30°C in the presence (+) or absence (-) of 1 mM IPTG. The indicated periplasm (20 μ l) was subjected to 13.5% SDS-PAGE in the presence of β -mercaptoethanol. Proteins in the gel matrix were electroblotted to nitrocellulose which was probed with 125 I-labeled anti-PT antibody. An autoradiogram of the probed nitrocellulose is shown. Numbers on right show molecular weight (K, 10^3).

Periplasmic extracts of IPTG-induced *E. coli* JM103 (ptacS-1c) and JM103(ptacC180) catalyzed the ADP-ribosylation of a 41,000-dalton protein (presumed to be the G_i protein [24]) in human erythrocyte membranes (Fig. 3). In the absence of added extracts or in the presence of extracts from IPTG-induced *E. coli* JM103(ptac18), ADP-ribosylation of the 41,000-dalton protein was not detected.

Subcellular localization of C180 peptide. The periplasmic fraction of IPTG-induced *E. coli* JM103(ptacC180) was en-

FIG. 3. ADP-ribosyltransferase activity in extracts of *E. coli* JM103. Protein (5 μ g) from the periplasmic fractions from *E. coli* JM103(ptacS-1c) (lane S1c), *E. coli* JM103(ptacC180) (lane C180), *E. coli*(ptac18) (lane 18), no extract (lane Bk), or urea-solubilized extract from *E. coli* LC137(pUCS-1term) (a positive control) (lane S1) was assayed for ADP-ribosyltransferase activity as described in Materials and Methods. The erythrocyte membranes in the reaction mixture were subjected to 12.5% SDS-PAGE in β -mercaptoethanol. An autoradiogram of the gel is shown. The size of the ADP-ribosylated proteins is given in kilodaltons on the left.

TABLE 1. Subcellular localization of C180 peptide in *E. coli* JM103(ptacC180)

Fraction ^a	Activity (%)		C180 peptide ^c (U)
	Glucose-6-phosphate dehydrogenase ^b	Alkaline phosphatase ^b	
Extracellular	ND ^d	ND	<1
Periplasmic	8	78	76
Cellular ^e	92	22	24

^a Subcellular fractionation was performed as described in Materials and Methods.

^b Glucose-6-phosphate dehydrogenase and alkaline phosphatase activities were determined as described in Materials and Methods and are reported as percent total activity.

^c C180 peptide was determined by subjecting the subcellular fractions to 13.5% SDS-PAGE in the presence of β -mercaptoethanol. Proteins in the gel matrix were electroblotted to nitrocellulose which was probed with ¹²⁵I-labeled anti-PT antibody. The amount (arbitrary units) of C180 peptide was determined by quantification of a densitometric scan of the autoradiogram.

^d ND, Not determined.

^e Amounts of marker enzymes and C180 peptide in the cytoplasmic and membrane fractions were added together and are represented as a single value.

riched for the C180 peptide (Table 1). Extraction of the periplasm was quantified with the periplasmic marker, alkaline phosphatase, and the cellular marker, glucose-6-phosphate dehydrogenase. The C180 peptide was quantified by densitometric scanning of autoradiograms of immunoblots of each fraction. Greater than 75% of the alkaline phosphatase activity and the C180 peptide were present in the periplasmic fraction, while <10% of the cellular marker, glucose-6-phosphate dehydrogenase, was present in this fraction. The cellular fraction was enriched for glucose-6-phosphate dehydrogenase; >90% of the glucose-6-phosphate dehydrogenase was present in this fraction, while it contained 22% of the alkaline phosphatase and 24% of the C180 peptide. The presence of these proteins in the cellular fraction may be attributed to incomplete formation of spheroplasts by lysozyme and/or incomplete release of spheroplast contents during fractionation. The periplasmic fraction of IPTG-induced *E. coli* JM103(ptacS-1c) was also similarly enriched for the rS-1 subunit (data not shown). We concluded that *E. coli* recognized the PT leader sequence of the S-1 subunit and secreted both rS-1 and the C180 peptide into the periplasm.

Purification of C180 peptide. The C180 peptide was purified to >90% homogeneity by a three-step protocol. A Coomassie blue protein stain and immunoblot of the pooled fractions of the purification are shown in Fig. 4 and are quantified in Table 2. The periplasmic extract from IPTG-induced *E. coli* JM103(ptacC180) was chromatographed over Sephacryl-S200 HR. The C180 peptide eluted as a single immunoreactive peak (labeled the S-200HR pool) which migrated slower than the peak protein fractions in the column effluent. Two peaks of NAD-glycohydrolase activity were identified in the column effluent. The slower-migrating peak of NAD-glycohydrolase activity coeluted with C180 peptide. The S-200 pool was subjected to DEAE chromatography. Following elution off the DEAE column, the C180 peptide was precipitated with 50% saturated ammonium sulfate (final concentration). This step both concentrated the C180 peptide and separated it from two contaminating proteins which coeluted with the C180 peptide during DEAE chromatography. The purified C180 peptide was determined to be >90% pure by densitometry of the Coomassie blue-stained SDS-polyacrylamide gel. The purified C180 peptide

FIG. 4. Purification of the C180 peptide. Equivalent cellular amounts of periplasmic extract (lanes A), S-200HR pool (lanes B), DEAE pool (lanes C), and ammonium sulfate-precipitated C180 peptide (lanes D) were subjected to 13.5% SDS-PAGE in the presence of β -mercaptoethanol. One half of the gel (1) was stained with Coomassie blue and is shown. The other half of the gel (2) was electroblotted to nitrocellulose which was probed with ¹²⁵I-labeled anti-PT antibody. An autoradiogram of the probed nitrocellulose is shown. Numbers on the left of panel 1 show size in kilodaltons.

retained full NAD-glycohydrolase activity following storage at 4°C for longer than 1 month. Under the growth conditions used, approximately 2.5 mg of C180 peptide was isolated from a 7-liter culture of cells.

Properties of C180 peptide. The fidelity of processing of the PT leader sequence by *E. coli* leader peptidase was determined by sequencing the C180 peptide. The N-terminus of the purified C180 peptide had the following sequence: Asp-Asp-Pro-Pro-Ala-Thr-Val-Tyr-Arg-Tyr-Asp-Ser-Arg-Pro-... No contaminating sequences were detected. The amino acid sequence of the C-180 peptide was identical to the predicted amino acid sequence of the S-1 subunit derived from the nucleotide sequence of the S-1 gene (14, 18) and the amino acid sequence determined by sequencing the N terminus of the S-1 subunit produced by *B. pertussis* (14, 18). A second peptide which eluted under higher salt concentration off the DEAE column relative to the C180 peptide was also observed (data not shown). This peptide represented about 5 to 10% of the immunoreactive material of the C180 peptide, migrated with the same molecular weight as the C180 peptide on reduced SDS-polyacrylamide gels, and possessed NAD-glycohydrolase activity. We speculate that this material represents improperly processed C180 peptide; future studies will resolve this possibility.

The amino acid composition of the C180 peptide was in close agreement with the amino acid composition derived

TABLE 2. Purification of C180 peptide from *E. coli*(ptacC180)

Fraction	Volume (ml)	Total protein (A ₂₈₀) ^a	C180 peptide (mg) ^b	Sp act (10 ³)	% Yield	Purification (fold)
Cells	7,000	ND ^c	5.5	ND	100	ND
Periplasmic fraction	175	1,698	4.3	2.5	78	1
S-200HR pool	160	251	3.2	12.8	58	5
DEAE pool	81	30	3.1	103.3	56	41
Ammonium sulfate (C180 peptide)	5	6	2.5	416.6	45	167

^a Total A₂₈₀ units.

^b Established by spectral analysis of the purified C180 peptide, using an extinction coefficient of 0.6 for a 10 μ M solution at 280 nm. Amount of C180 peptide in the earlier steps of purification was determined by quantification of immunoreactive material in these fractions relative to purified C180 peptide.

^c ND, Not determined.

TABLE 3. Amino acid composition of C180 peptide^a

Residue	mol% (SD)	Residues/C180 exptl ^b	Residues/C180 calculated ^c
Asx	12.4 (0.11)	22.1	22
Glx	10.9 (0.23)	19.5	18
Arg	8.7 (0.50)	15.6	17
Ala	9.0 (0.04)	16.1	16
Tyr	8.3 (0.61)	14.9	16
Thr	7.8 (0.23)	13.9	16
Ser	5.8 (0.23)	10.4	15
Gly	7.9 (0.04)	14.1	14
Val	7.4 (0.16)	13.3	12
Pro	4.8 (0.07)	8.5	8
Ile	4.4 (0.05)	7.8	7
Leu	5.0 (0.45)	8.9	6
Phe	2.9 (0.02)	5.2	6
His	2.6 (0.07)	4.6	5
Met	0.9 (0.19)	1.7	1
Lys	1.4 (0.44)	1.4	0

^a The amino acid composition of two independent preparations of the C180 peptide was determined following hydrolysis in 6 N HCl with a Beckman 6300 amino acid analyzer.

^b Fractional moles percent of one preparation of C180 peptide $\times$ 181 residues.

^c From reference 18.

from the nucleotide sequence of the DNA encoding for the S-1 gene (Table 3). Combining these data and UV spectral analysis, we determined the extinction coefficients for a 10 μ M solution of C180 peptide to be 2.5 and 0.6 absorbance units at 230 and 280 nm, respectively. The UV spectrum of the C180 peptide identified an absorbance maximum at 276 nm, a minimum at 251 nm, and a 280 nm/260 nm absorbance ratio of 1.41.

Properties of anti-C180 IgG. IgG fractions were prepared from preimmunized sera (preimmune) and immunized sera (immune) of rabbits challenged with the C180 peptide. Immunoblotting with these IgG fractions showed that while the preimmune IgG did not react with C180 peptide, the IgG fraction purified from sera of immunized animals reacted

with the peptide (data not shown). Preincubation of the C180 peptide with the IgG fraction from immunized sera resulted in an inhibition of NAD-glycohydrolase activity of the C180 peptide that depended on the concentration of IgG (data not shown). Preincubation with an equivalent amount of IgG from preimmunized sera did not affect enzyme activity.

NAD-glycohydrolase activity of two mutants of C180 peptide. Single amino acid substitutions within the S-1 subunit which reduced ADP-ribosyltransferase activity have been identified by us and other investigators (1, 6, 20). Our studies have focused on defining the function of two amino acid residues of the S-1 subunit, tryptophan 26 and glutamic acid 139. Our earlier studies showed that substitution of either tryptophan 26 with isoleucine or glutamic acid 139 with serine reduced ADP-ribosyltransferase activity of the S-1 subunit (1). However, owing to endogenous NAD-glycohydrolase activity in the cell extracts, further characterization of the effect that these substitutions had on enzyme activity was not determined. To extend these studies, we introduced gene fragments containing each substitution into ptacC180, generating C180I-26 and C180S-139. C180I-26 possessed a reduced level of NAD-glycohydrolase activity relative to the C180 peptide. During the first 20 min of incubation, the rate of NAD-glycohydrolase activity of C180I-26 was approximately 1% that of the C180 peptide (Fig. 5). In contrast, C180S-139 possessed essentially the same level of NAD-glycohydrolase activity as the C180 peptide.

DISCUSSION

The inability of *E. coli* to transcribe the PT promoter (19) has prompted several laboratories to express the subunits of PT as gene fusions under the regulation of *E. coli* promoters (2, 5, 13, 17). The gene products of these constructions have been used to localize functional regions of the S-1 subunit (1, 3, 13, 20). In this study, we expressed the genes encoding for rS-1 and the C180 peptide under the regulation of the *tac* promoter as a nonfusion gene product. *E. coli* localized both rS-1 and the C180 peptide as soluble, secreted peptides with correct processing of the PT leader sequence. The *tac*

FIG. 5. NAD-glycohydrolase activity of the wild-type C180 peptide (WT) and mutants of the C180 peptide. C180 peptide equivalents were purified from IPTG-induced *E. coli* JM103(ptacC180), JM103(ptacC180I-26), or JM103(ptacC180S-139). NAD-glycohydrolase activity was assayed for the indicated times as described in Materials and Methods. The reaction mixtures contained 10.9 μ M [³H]NAD plus 0.7 μ M C180 peptide (●), C180I-26 peptide (■), or C180S-139 peptide (▲). The results are presented as the average of an experiment performed in duplicate.

promoter was chosen as the surrogate promoter for the S-1 gene because it is efficiently induced with IPTG in a *lacI^q* host. This expression system has been used by others to express *Pseudomonas* exotoxin A (10) and diphtheria toxin (Collier et al., in preparation) as well as other heterologous proteins (10) in *E. coli*. The protocol used to insert the S-1 subunit into *ptac18* may prove useful for the construction of expression vectors of other genes, since the synthetic oligonucleotide cassette used in our construction allowed proper nucleotide composition and spacing with respect to the promoter. Burnette et al. (7) have also expressed individual subunits of PT as nonfusion proteins in *E. coli* under the regulation of the bacteriophage lambda promoter. These constructs were expressed at high levels when the native PT leader sequence was inserted between the promoter and the structural gene. The gene products were delivered to inclusion bodies and could be solubilized with urea. Although speculative, the difference in the subcellular localization between the gene products expressed with the *tac* expression system (periplasm) and the lambda expression system (inclusion body) may reflect the level of gene expression achieved with each vector or may be due to the growth conditions or induction protocol used for the expression of these recombinant proteins.

The S-1 subunit of PT has also been expressed in *B. subtilis* (21). This expression system aligned DNA encoding the mature S-1 subunit, except the first amino acid, downstream from the signal sequence and first amino acid codon of α -amylase of *Bacillus amyloliquefaciens*. Large amounts of S-1 subunit were expressed and secreted into the culture medium, although degradation of the S-1 subunit was observed. This expression system may be useful for the expression of mutant forms of PT which have been produced in *E. coli* (1, 6, 20). In *B. pertussis*, it appears that the individual subunits of PT are secreted into the periplasm, where the subunits assemble and are then exported into the culture medium (19). It will be interesting to see how *B. subtilis*, a gram-positive bacterium which lacks a periplasm, will assemble the multiple subunits of PT.

The fact that the rS-1 and C180 peptides were secreted into the periplasm of *E. coli* argues against the need for auxiliary *B. pertussis* proteins or the other PT subunits for translocation of the S-1 subunit across the cytoplasmic membrane. The leader sequence of the S-1 subunit appears to direct the periplasmic localization of the structural gene. Consistent with the role of the leader sequence in protein localization were the observations that the products of gene fusions containing the S-1 subunit without the PT leader sequence were localized to the cytoplasm (2, 13, 17). It was not unexpected that the 34-amino-acid S-1 leader sequence was recognized in *E. coli* since the overall structure of the PT leader sequence conforms to the consensus structure of bacterial leader sequences and since there is considerable divergency in the amino acid sequences that can function as leader sequences (25). Upon traversing the cytoplasmic membrane, the junction between the leader sequence and the mature rS-1 subunit and C180 peptide was recognized by a leader peptidase and correctly processed.

The purified C180 peptide expressed both NAD-glycohydrolase and ADP-ribosyltransferase activities. These results are consistent with our earlier mapping studies which showed that amino acids 2 through 180 of the S-1 subunit were required for expression of enzymatic activity (1). The C180 peptide proved to be an effective immunogen. Purified IgG from immunized animals neutralized catalytic activity (NAD-glycohydrolase). Future studies will determine

whether this neutralization was due to allosteric association of the IgG to the peptide or due to a specific interaction between the IgG and an epitope contained within the NAD-binding site.

Although the mature form of the S-1 subunit was detected in the periplasm of IPTG-induced *E. coli*(*ptacS-1c*), several lower-molecular-weight immunoreactive peptides were detected. These peptides are apparently proteolytic degradation products of rS-1. Future experiments will determine whether culture conditions can be established to reduce the degradation of rS-1. Another approach to eliminating proteolysis of the S-1 subunit might be to express the S-1 subunit and the other subunits of PT as a polycistronic operon in *E. coli*, since isolated S-1 subunit has been shown to be more susceptible to proteolysis than S-1 subunit complexed within PT (8). Although many problems, both technical and biological, may hamper the contiguous expression of the five genes encoding for PT in *E. coli*, two recent studies have reported that heterologous proteins expressed as a dicistronic operon correctly assemble within the periplasm (23) and culture medium (4) of *E. coli*.

In an earlier study, we identified several single amino acid substitutions within the S-1 subunit which reduced the ADP-ribosyltransferase activity (1). Owing to endogenous levels of NAD-glycohydrolase activity in the cell extracts, we were unable to measure the NAD-glycohydrolase activity in the cell extracts of these mutants. Thus, the role that these residues had on enzyme activity was not determined. We focused our studies on defining the role that two of these amino acids, tryptophan 26 and glutamic acid 139, play in the ADP-ribosyltransferase reaction. Therefore, gene fragments were transferred from the pUC-derived expression vectors (1) into *ptacC180*, which allowed production of these mutants in a secreted, nonfusion form amenable to purification. C180I-26 possessed approximately 1% of the NAD-glycohydrolase activity of the C180 peptide. These results suggest that tryptophan 26 plays a role in the interaction, either binding or hydrolysis, of NAD with the C180 peptide. Conversely, C180S-139 possessed NAD-glycohydrolase activity equivalent to that of the C180 peptide. These results indicate that glutamic acid 139 does not play a role in the interaction between NAD and the peptide but functions at a step in the ADP-ribosyltransferase reaction subsequent to NAD binding and hydrolysis.

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