Biochemical Analysis of Mutations at Tyrosine-98 of the S1 Subunit of Pertussis Toxin†

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ABSTRACT: Molecular modeling and alignment of the primary amino acid sequence of the S1 subunit (S1) of pertussis toxin (PT) with other members of the family of ADP-ribosylating bacterial exotoxins predicted that tyrosine-98 (Y98) of S1 was a conserved residue among these exotoxins. To extend our understanding of the relationship between pertussis toxin and the other ADP-ribosylating exotoxins, we defined the function of Y98 of S1. Using site-directed mutagenesis, Y98 of S1 was substituted with alanine (Y98A), leucine (Y98L), histidine (Y98H), and phenylalanine (Y98F). Mutations were analyzed in the C180 peptide and C219 peptide, recombinant derivatives of S1 which contain the first 180 and 219 amino-terminal residues of S1, respectively. Periplasmic extracts containing the Y98*n* peptides expressed similar specific activities for the ADP-ribosylation of transducin (G_t) as the periplasmic extract containing wild-type peptides. Mutations at Y98 influenced the subcellular localization of the respective Y98*n* peptide. The majority of the wild-type Y98 and Y98F peptides localized to the periplasmic extract, while the majority of Y98A and Y98L peptides were associated with the insoluble bacterial outer membrane. Purified C180Y98A and C180Y98F and partially purified C180Y98H peptides possessed similar specific activities for the ADP-ribosylation of G_t as the wild-type C180 peptide. K_{mNAD} and k_{cat} for C180Y98A and C180Y98F in the NAD glycohydrolase reaction were similar to the wild-type C180 peptide. These data show that the R group of Y98 does not participate in the ADP-ribosylation of G_t, but appears to contribute to the proper folding of S1.

Pertussis toxin (PT; molecular mass 105 060 daltons) is a member of a family of bacterial exotoxins that catalyze the transfer of the ADP-ribose portion of NAD to specific target proteins in eukaryotic cells (Tamura, 1982). PT is composed of six noncovalently bound subunits designated S1 through S5 which are organized in a 1:1:1:2:1 ratio, respectively. PT follows the "A:B" model for exotoxin structure–function (Gill, 1978). The "A" component (S1) possesses enzymatic activity, and the "B" component (S2 through S5) binds to cell-surface receptors to enable S1 to enter the cell. Upon entry into the cell, the S1 subunit catalyzes the transfer of the ADP-ribose portion of NAD to a cysteine residue located near the carboxyl terminus of the α subunit of several heterotrimeric G proteins, including G_i, G_o, and G_t. ADP-ribosylation uncouples signal transduction between the G proteins and their respective G protein coupled receptors (Hsia, 1984). In the absence of G protein, PT catalyzes the hydrolysis of NAD to ADP-ribose and nicotinamide (Katada, 1983; Moss, 1983). Although the *in vivo* role of this reaction is not apparent, analysis of this reaction is useful for the measurement of enzyme activity. The goal of the structure–function analysis of PT is to provide information to be used for the rational approach of engineering a recombinant toxoid of PT to be used in the development of an acellular vaccine against infection by *Bordetella pertussis* (Barbieri, 1988; Cieplak, 1988; Locht, 1989; Pizza, 1988, 1989; Loosemore, 1990).

The operon encoding the PT subunits has been cloned and sequenced (Locht, 1986b; Nicosia, 1986), and the individual subunit genes have been expressed in *Escherichia coli* as both fusion and nonfusion proteins (Barbieri, 1987; Burnette, 1988; Locht, 1987; Nicosia, 1987). The functional domains of S1 (235 amino acids) have been determined. NAD binding and hydrolysis reside within the amino-terminal 180 residues of S1, represented by a recombinant derivative of S1 termed the C180 peptide. The complete ADP-ribosyltransferase activity resides within the amino-terminal 219 residues of S1, represented by a recombinant derivative of S1 termed the C219 peptide (Cortina, 1989).

Several earlier studies have predicted a functional role for Y98 of S1 in the ADP-ribosylation reaction. Computer modeling of S1 of PT and diphtheria toxin (DT) with the solved crystal structure of exotoxin A of *Pseudomonas aeruginosa* (ETA) (Domenighini, 1991) predicted that Y98 of S1 and Y65 of DT were homologous to Y481 of ETA and would lie within the NAD binding domain of their respective toxins. To determine the functional role of Y98 of S1 and its relationship to Y481 of exotoxin A and Y65 of DT, both conservative and nonconservative amino acid substitutions for Y98 in the S1 subunit of PT were engineered. Our studies show that the R group of Y98 does not participate in the ADP-ribosylation of G_t, but appears to be required for proper protein folding.

MATERIALS AND METHODS

Materials. Construction of ptac18, ptacC180, ptacC219, and M13mp18C180 has been described previously (Barbieri, 1988, 1989; Cortina, 1989). Oligonucleotides were synthesized at the Shared Research Facility of the Medical College of Wisconsin. An oligonucleotide-directed *in vitro* mutagenesis system was obtained from Amersham Corp. Plasmid DNA was prepared using the Plasmid Medi Kit from Qiagen (Germany). DNA sequencing was performed using the Sequenase DNA-sequencing kit (United States Biochemical Corp., Cleveland, OH). Restriction endonucleases and T4

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DNA ligase were purchased from either International Biotechnologies (New Haven, CT) or New England BioLabs (Beverly, MA). [α-^{32}P]dATP and [*adenylate phosphate*-^{32}P]NAD were purchased from Du Pont–New England Nuclear. [^{125}I]Iodine and [*nicotinamide*-^{3}H]NAD were purchased from Amersham Corp. Protein molecular mass markers were purchased from Pharmacia–LKB Biotechnology Inc. Analysis of kinetic data was performed with data analysis software Enzfitter (Elsevier–Biosoft, Cambridge, United Kingdom).

Site-Directed Mutagenesis. The *in vitro* oligonucleotide-directed mutagenesis method (Taylor, 1985), as marketed by Amersham International, was used to produce mutations at residue 98 of S1. An *Eco*RI–*Hin*dIII DNA fragment encoding the wild-type C180 structural protein was cloned into M13mp18, yielding M13mp18C180. Single-stranded phage DNA from M13mp18C180 was used as the mutagenesis template. Oligonucleotides used to generate single amino acid substitutions at residue 98 of the C180 peptide are as follows (mutated bases are underlined and in *italic*): Y98A, 5′TGG CGG CGC C*ag* cGA AAT TGT TG3′; Y98F, 5′CGG CGC CG*a* AGA AAT TGT T3′; Y98H, 5′CGG CGC CGT *g*GA AAT TGT T3′; Y98L, 5′GCG GCG CCG *ag*G AAA TTG TT3′. Mutations were confirmed by DNA sequencing of the C180 gene. Double-stranded DNA of the replicative form of the M13 phage harboring the mutated C180 gene was isolated and digested with *Bam*HI and *Sal*I. The 444 bp *Bam*HI–*Sal*I DNA fragments were ligated into either a ptacC180 or a ptacC219 vector which had been digested with the same restriction enzymes and transformed into competent TG1 cells. Plasmids were isolated and subcloned into the *deg P* and *omp T* protease-deficient *E. coli* strain SK110 which was the host strain used for the expression of the C180Y98*n* peptides or C219Y98*n* peptides.

Measurement of Steady-State Expression Levels of C180Y98n Peptides. Whole cell lysate were prepared as follows: overnight cultures of *E. coli* harboring one of the ptacC180Y98*n* plasmids were diluted 1/100 in 3 mL of L-broth supplemented with 100 μg of ampicillin/mL and 0.5 mM $ZnSO_4$ and incubated at 250 rpm at 30 °C. After 6 h, cell cultures (0.4 mL) were centrifuged at 13800*g* for 2 min, resuspended in 40 μL of SDS–PAGE sample buffer containing β-mercaptoethanol, and boiled for 5 min. Equal amounts of samples (cell culture equivalents were within 10%) were subjected to SDS–PAGE followed by immunoblotting using α-C180 peptide IgG. A duplicate gel was stained with Coomassie blue to determine the amount of protein loaded in each lane. The amount of immunoreactive peptide was normalized to the amount of protein loaded in each lane. Preparation of α-C180 peptide IgG has been previously described (Barbieri, 1989).

The steady-state expression of wild-type C180 and C180Y98A was also measured following growth at 20 °C. This experiment was performed as described for the measurement of steady-state expression at 30 °C, except that overnight cultures were grown at 20 °C and that, following dilution of the overnight culture into L-broth, cells were incubated at 20 °C, until the A_{600} reached 0.5.

Subcellular Fractionation of E. coli. Cultures were prepared as described above except that 30-mL cultures were grown and fractionated to obtain periplasmic, cytosolic, inner membrane, and outer membrane fractions (Barbieri, 1989). Separation of the periplasmic extract was performed by spheroplast formation using a hypertonic lysozyme treatment (Barbieri, 1989). Lysozyme-treated cells were resuspended in 0.5 mL of 25 mM Tris-HCl (pH 7.6) containing 100 mM NaCl, 1 mM $MgCl_2$, and 10 μg of DNase I/mL and broken by freezing/thawing 5 times in a dry ice–ethanol bath. The cytoplasmic and membrane components were separated by centrifugation at 13800*g* for 15 min at 4 °C. The pellet, containing the total membrane fraction, was resuspended in 0.5 mL of 25 mM Tris-HCl (pH 7.6) containing 0.1% SDS. To separate the inner membrane and outer membrane, the pellet which contained the total membrane fraction was resuspended in 0.5 mL of 10 mM Tris-HCl (pH 7.6) containing 100 mM NaCl and 2% Triton X-100. Inner membranes (soluble) and outer membranes (insoluble) were separated by centrifugation at 13800*g* for 15 min at 4 °C. The insoluble material containing the outer membranes was resuspended in 0.5 mL of 25 mM Tris-HCl (pH 7.6) containing 0.1% SDS. Equal cellular amounts of the periplasmic, cytoplasmic, inner membrane, and outer membrane fractions were subjected to SDS–PAGE followed by Western blot analysis by probing with α-C180 peptide IgG and ^{125}I-protein A. Quantitation was performed by densitometry of the autoradiograms. Subcellular fractionation was performed on cells cultivated at both 20 °C and 30 °C.

Purification of C180Y98n Peptides. The plasmid vectors encoding the C180Y98*n* peptides were engineered to include the S1 signal sequence immediately amino terminal to the C180 and C219 peptide sequences (Barbieri, 1989). This leader sequence was included to deliver the C180Y98*n* and C219Y98*n* peptides to the periplasmic fraction. However, subcellular fractionation showed that mutations at residue 98 influenced the distribution of their respective peptide. This required the development of an individual strategy for the purification of each recombinant protein.

(*a*) *Wild-Type C180 Peptide.* Purification of the wild-type C180 peptide from *E. coli* has been described (Barbieri, 1989). Briefly, 6 L of *E. coli*(ptacC180) was sparged with oxygen for 6–8 h in the presence of 100 μg of ampicillin/mL and 0.5 mM $ZnSO_4$, and a periplasmic extract was prepared by hypertonic lysozyme treatment. The periplasmic extract was adjusted to a final concentration of 50% ammonium sulfate and stored at 4 °C overnight. The precipitate was concentrated, resuspended in 25 mM Tris-HCl (pH 7.6) containing 10 mM β-mercaptoethanol, and subjected to Sephacryl S-200 HR chromatography. Column fractions containing the C180 peptide were pooled and chromatographed over DEAE-Sephacryl with a linear gradient between 50 and 200 mM NaCl. Column fractions containing the C180 peptide were pooled and concentrated by ammonium sulfate precipitation (50% final concentration). The resulting material was termed the wild-type C180 peptide.

(*b*) *C180Y98F Peptide.* Overnight cultures of *E. coli*(ptacC180Y98F) were diluted 1/50 into 2 L of L-broth supplemented with 100 μg of ampicillin/mL and 0.5 mM $ZnSO_4$ and incubated at 250 rpm at 30 °C for 5 h. The C180Y98F peptide was purified from the periplasmic extract as described for the wild-type C180 peptide, except that the DEAE ion-exchange chromatography step was omitted.

(*c*) *C180Y98A Peptide.* Overnight cultures of *E. coli*(ptacC180Y98A) were diluted 1/50 into 2 L of L-broth supplemented with 100 μg of ampicillin/mL and 0.5 mM $ZnSO_4$ and incubated at 250 rpm at 30 °C for 5 h. The C180Y98A peptide was purified as follows at 4 °C. Cells were concentrated, washed with 30 mL of 25 mM Tris-HCl (pH 7.6), and resuspended in 20 mL of 25 mM Tris-HCl (pH 7.6) containing 100 μL of 2 mg of RNase/mL and 100 μL of 2 mg of DNase I/mL. Cells were broken by French press (2 times), and

unbroken cells were pelleted by centrifugation at 3000*g* for 5 min. Broken cells were subjected to centrifugation at 43000*g* for 30 min. The particulate membrane fraction was suspended in 10 mM Tris-HCl (pH 7.6) buffer containing 1 mM $MgCl_2$ and 2% Triton X-100 and centrifuged at 43000*g* for 30 min. The Triton X-100 extraction step was repeated once. The particulate material was washed with 20 mL of 25 mM Tris-HCl (pH 7.6) containing 1 M NaCl. The particulate material was suspended in 5 mL of 25 mM Tris-HCl (pH 7.6) containing 8 M urea and incubated overnight at 4 °C. This 8 M urea extract was subjected to ultracentrifugation in a Ti75 rotor at 40 000 rpm for 3 h. The soluble material was subjected to Sephacryl S-200 HR chromatography using 25 mM Tris-HCl (pH 7.6) containing 6 M urea as the elution buffer. Column fractions containing the C180Y98A peptide were pooled and precipitated with ammonium sulfate (50% final concentration). The precipitate was concentrated and suspended in 25 mM Tris (pH 7.6) containing 2 M urea and termed the C180Y98A peptide. N-Terminal sequencing of the C180Y98A peptide showed that the S1 signal sequence had been properly processed (Xu & Barbieri, unpublished data).

(*d*) *C180Y98H Peptide.* Overnight cultures of *E. coli*(ptacC180Y98H) were diluted 1/50 into 2 L of L-broth supplemented with 100 μg of ampicillin/mL and 0.5 mM $ZnSO_4$ and incubated at 250 rpm at 30 °C for 5 h. A periplasmic extract was prepared by hypertonic lysozyme treatment. The periplasmic extract was adjusted to a final concentration of 50% saturated ammonium sulfate and stored at 4 °C overnight. The precipitate was concentrated and suspended in 25 mM Tris-HCl (pH 7.6) containing 10 mM β-mercaptoethanol and subjected to Sephacryl S-200 HR chromatography. Column fractions containing the C180Y98H peptide were pooled and concentrated by ultrafiltration using a YM-3 membrane (Amicon, molecular weight cutoff = 3000). This material was termed the C180Y98H peptide.

ADP-Ribosyltransferase Activities of C180Y98n Peptides. The reaction contained the following in 20 μL: 0.1 M Tris-HCl (pH 7.6), 20 mM dithiothreitol (DTT), 0.1 μM [*adenylate phosphate*-^{32}P]NAD (specific activity 100 Ci/mmol), 0.1 μM transducin (G_t), and an aliquot of a periplasmic extract or purified peptide. The assay was performed at room temperature and terminated at the appropriate time by the addition of SDS sample buffer containing β-mercaptoethanol and boiled for 5 min. Samples were subjected to SDS–PAGE. The gel was dried and exposed to X-ray film. Quantitation of the autoradiograms was performed with an AMBIS optical imaging system. The peptide corresponding to the α-subunit of G_t was then cut out of the dried gel, and radioactivity was determined directly in a Beckman LS 6000IC scintillation counter.

NAD Glycohydrolase Activity of C180Y98n Peptides. The reaction contained the following in 25 μL: 0.1 M Tris-HCl (pH 7.6), 1.0 mg of egg albumin/mL, 20 mM DTT, varied amounts of [*nicotinamide*-^{3}H]NAD, and an aliquot of purified peptide. The assay mixture was incubated at room temperature. At the appropriate time, the reaction was terminated by the addition of 10 μL of 1.0 M sodium borate (pH 8.0). Hydrolyzed nicotinamide was separated from NAD by the addition of 225 μL of water-saturated ethyl acetate. The amount of nicotinamide in the ethyl acetate phase was determined by liquid scintillation counting. The rate of NAD glycohydrolase activity (picomoles of NAD released per hour) was plotted against the concentration of substrate (micromolar NAD). A double-reciprocal plot of these data yielded K_{mNAD} and V_{max} values. C180Y98*n* peptide concentrations were determined by subjecting the purified C180Y98*n* peptides, along with known concentrations of wild-type C180 peptide, to SDS–PAGE followed by staining of the proteins with Coomassie blue and quantitation with an AMBIS optical imaging system. The calculated amount of each C180Y98*n* peptide was used to normalize the V_{max} value to obtain the k_{cat}.

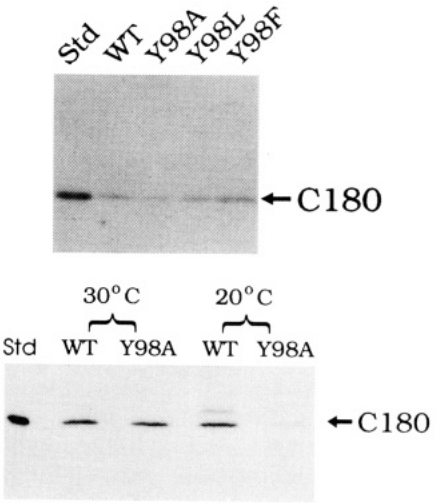

FIGURE 1: Steady-state expression of C180Y98*n* peptides in *E. coli* cultivated at 30 or 20 °C. Overnight cultures of *E. coli* ptacC180 (WT), ptacC180Y98A (Y98A), ptacC180Y98L (Y98L), or ptacC180Y98F (Y98F) grown at 30 or 20 °C were diluted 1/100 in 3 mL of L-broth supplemented with 100 μg of ampicillin/mL and 0.5 mM $ZnSO_4$ and incubated at 250 rpm at 30 or 20 °C. After the A_{600} reached 0.5, cell cultures were centrifuged, resuspended in SDS–PAGE sample buffer containing β-mercaptoethanol, and boiled. Equal cell equivalents (within 10%) were subjected to SDS–PAGE followed by immunoblotting using α-C180 peptide IgG and ^{125}I-protein A. A photograph of the autoradiogram is shown. (Top panel) Steady-state expression levels of C180Y98*n* peptides at 30 °C. (Bottom panel) Steady-state expression levels of C180WT and C180Y98A peptides at 30 and 20 °C. The leftmost lane (Std) contains 0.03 μg of a standard preparation of the C180 peptide. Arrows indicate the migration of the C180 peptide.

Preparation of G_t. G_t was prepared according to a published purification protocol (Baehr, 1982). The purified G_t was suspended in 0.1 mM DTT, 10 mM Tris-HCl (pH 7.6), and 40 μM GTP, concentrated 5-fold by ultrafiltration (Amicon YM-5), and stored at −20 °C in 40% glycerol. A determination of the total ADP-ribosylable G_t was made by incubating an aliquot of G_t with excess NAD and rS1 and extrapolating for the amount of G_t ADP-ribosylated from a first-order rate projection.

RESULTS

Steady-State Expression of C180Y98n Peptides at 30 °C. *E. coli* harboring ptacC180Y98F, ptacC180Y98A, or ptacC180Y98L expressed a single peptide which reacted with S1-specific IgG and comigrated with the wild-type C180 peptide (Figure 1, top panel). The steady-state expressions of C180Y98F, C180Y98A, and C180Y98L were similar (within 20%) to the steady-state expression of the wild-type C180 peptide. In these experiments, steady-state expression represents the amounts of C180Y98*n* peptide present in the cells prior to subcellular localization. In a separate experiment, analysis of *E. coli* harboring ptacC180Y98H showed that C180Y98H was also expressed at a steady-state level similar to the wild-type C180 peptide (data not shown).

ADP-Ribosylation Activities of C180Y98n Peptides in Periplasmic Extracts. Periplasmic extracts were prepared

Table 1: ADP-Ribosyltransferase Activity in Periplasmic Extracts of *E. coli* and of Purified C180 Peptides

	peptide analyzed			
	WT	Y98A	Y98L	Y98F
Periplasmic Extract of 180 Peptides				
ADP-ribosyltransferase act. (units)[a]	54.0	3.2	8.8	62.0
immunoreactive peptide (units)[b]	12.0	2.7	3.2	17.0
sp act.[c]	4.5	1.2	2.8	3.6
Periplasmic Extract of C219 Peptides				
ADP-ribosyltransferase act. (units)[a]	102.4	12	9.9	94.7
immunoreactive peptide (units)[b]	54.8	8.0	5.5	51.6
sp act.[c]	1.9	1.5	1.8	1.8
Purified C180 Peptides				
sp act.[d]	1.8	1.2	1.4	2.0

[a] ADP-ribosyltransferase activity units. Reaction mixtures were subjected to SDS–PAGE following by autoradiography. The autoradiographic signal of the α-subunit of G_t was determined by densitometry. [b] Immunoreactive peptide units. Periplasmic extracts were subjected to SDS–PAGE followed by Western blot analysis using α-C180 peptide IgG and ^{125}I-protein A. The autoradiographic signals of Western blots were determined by densitometry, and units of immunoreactive peptides were given arbitrary unit values. [c] Specific ADP-ribosyltransferase activity was reported as ADP-ribosyltransferase activity units per immunoreactive peptide unit. [d] Specific ADP-ribosyltransferase activity was reported as ADP-ribosyltransferase activity units per minute per picomole of peptide.

from *E. coli* harboring ptacC180Y98*n*. Each periplasmic extract was evaluated for the ability to ADP-ribosylate G_t and for the presence of C180 peptide immunoreactive material. Periplasmic extracts were analyzed because the expression vectors were engineered to include the S1 signal sequence which should direct the secretion of the C180Y98*n* peptides to the periplasm of *E. coli* (Barbieri, 1989). The specific ADP-ribosyltransferase activities of the periplasmic extracts possessing C180Y98F, C180Y98L, and C180Y98A were 80, 60, and 30%, respectively, of that observed for the periplasmic extract possessing the wild-type C180 peptide (Table 1). Periplasmic extracts from *E. coli* harboring vector alone (ptac18) did not possess detectable amounts of ADP-ribosyltransferase activity. The results shown in Table 1 are from one set of data reflecting a representative experiment.

Subcellular Localization of C180Y98n Peptides at 30 °C. Although the specific ADP-ribosyltransferase activities of the C180Y98*n* peptides in the periplasmic extracts were similar, the absolute amount of each respective C180Y98*n* peptide differed within each periplasmic extract (Table 1). Since the steady-state levels of the C180Y98*n* peptides were similar (Figure 1, top panel), this was interpreted to mean either that the C180Y98*n* peptides were being degraded in the periplasmic extract or that some of the C180Y98*n* peptides were not localized within the periplasmic extract. *E. coli* harboring ptacC180Y98*n* were subjected to subcellular fractionation, and each fraction was analyzed for the presence of a C180-immunoreactive peptide. As shown in Figure 2, >75% of the wild-type C180 and C180Y98F peptides localized to the periplasmic fraction, indicating that the removal of the phenolic OH of the R group did not influence C180 peptide localization. In contrast, only a small fraction of the C180Y98A and C180Y98L peptides localized to the periplasmic extract, with the majority of these peptides localizing to the insoluble membrane fraction. Further separation of the membrane fraction into inner and outer membrane components showed that the majority of the C180Y98A and C180Y98L peptides were associated with the insoluble outer membrane fraction. The C180Y98H peptide showed an intermediate subcellular localization relative to the other C180Y98*n* peptides. The results of the subcellular fractionation have been summarized

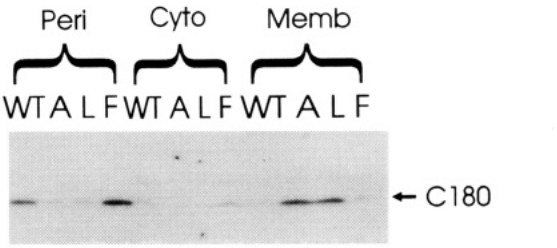

FIGURE 2: Subcellular localization of C180Y98*n* peptides expressed in *E. coli*. Overnight cultures (30 mL) of *E. coli* ptacC180WT (WT), ptacC180Y98A (A), ptacC180Y98L (L), or ptacC180Y98F (F) were grown as describe in Figure 1 and processed to obtain the periplasmic, cytosolic, and membrane fractions as described under Materials and Methods. Equal cellular amounts of each fraction were subjected to SDS–PAGE followed by Western blot analysis by probing with α-C180 peptide IgG and ^{125}I-protein A. A photograph of the autoradiogram is shown. The left lane (Std) contains 0.03 μg of a standard preparation of the C180 peptide. The arrow indicates the migration of the C180 peptide.

Table 2: Subcellular Localization of C180Y98*n* Peptides in *E. coli* Cultivated at 30 or 20 °C

C180 peptide at	% of total immunoreactive peptide localized in[a]			
	periplasm	cytoplasm	inner membrane	outer membrane[b]
30 °C				
WT	84	16	0	0
Y98A	27	2	0	71
Y98L	26	2	6	66
Y98H	63	0	4	33
Y98F	77	19	0	4
20 °C				
WT	83	12	1	3
Y98A	50	0	4	46

[a] The percentage of immunoreactive peptide was determined as [(immunoreactive peptide in individual fraction)/(immunoreactive peptide in all fractions)] × 100. [b] The outer membrane fraction represents the amount of immunoreactive material remaining after the fractionation protocol.

in Table 2. The fact that C180Y98A, C180Y98L, and C180Y98H localized to a subcellular fraction different than the wild-type C180 peptide implicated a role for residue 98 in either the folding or the maintenance of conformation of the C180 peptide.

Influence of Temperature on the Steady-State Expression and Subcellular Distribution of Wild-Type C180 and C180Y98A. Single amino acid substitutions have been shown to influence both the folding and stability of recombinant proteins (Matthews, 1983; Yu, 1984; Bachinger, 1993; Luo, 1993). To determine whether temperature influenced production of the Y98A mutant, *E. coli* harboring ptacC180 or ptacC180Y98A were cultivated at 20 °C and analyzed for steady-state expression and subcellular localization of the C180 peptide. Steady-state expression and subcellular distribution of wild-type C180 were similar following cultivation at either 20 °C or 30 °C (Figure 1, bottom panel, and Table 2). In contrast, C180Y98A was expressed at a lower steady-state level at 20 °C (14% of that at 30 °C) with an increase in the proportion of C180Y98A localized in the periplasmic fraction (Figure 1, bottom panel, and Table 2). The response of the Y98A mutation to temperature, although indirect, was consistent with residue 98 playing a role in the proper folding of the C180 peptide.

ADP-Ribosylation Activities of C219Y98n Peptides in Periplasmic Extracts. The Y98 substitutions were also introduced into a second derivative of S1, the C219 peptide.

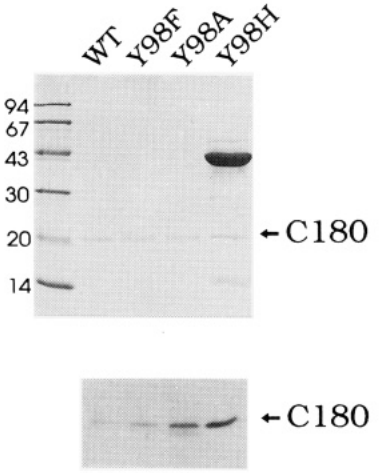

FIGURE 3: SDS–PAGE and immunoblotting of the purified C180Y98*n* peptides. Wild-type C180 peptide (WT), C180Y98F (Y98F), C180Y98A (Y98A), and C180Y98H (Y98H) were purified as described under Materials and Methods. Aliquots of the resulting materials of each purification were subjected to 13.5% SDS–PAGE, and the gels were stained for protein with Coomassie blue (upper panel) or subjected to immunoblotting using α-C180 IgG followed by ^{125}I-protein A (lower panel) as described under Materials and Methods. Upper panel: migration of molecular mass standards in the leftmost lane is indicated in the left margin in kilodaltons. Arrows indicate the migration of the C180 peptide.

C219 contains the amino-terminal 219 amino acid residues of the S1 subunit and appears to have the same ADP-ribosyltransferase and NAD glycohydrolase activities as the S1 subunit (Cortina, 1991), but is secreted into the periplasmic fraction of *E. coli* as a more soluble protein than S1. C219 is not routinely used for the analysis of residues thought to function in catalysis, since the C219 peptide is expressed at a lower steady-state level than the C180 peptide. Analysis of ADP-ribosyltransferase activity within periplasmic extracts containing C219Y98F, C219Y98A, and C219Y98L showed that each C219Y98*n* peptide possessed similar specific activities for ADP-ribosylation of G_t as extracts containing wild-type C219 peptide (Table 1). Analysis of the subcellular distribution of the C219Y98*n* peptides showed that, like the C180Y98*n* peptides, the majority of the C219Y98F and wild-type C219 peptides localized to the periplasmic extract while the majority of the C219Y98A and C219Y98L peptides localized within the insoluble bacterial outer membrane fraction (data not shown).

ADP-Ribosylation of G_t by Purified C180Y98n Peptides. The C180Y98*n* peptides were purified as described under Materials and Methods. The C180Y98F and C180Y98A peptides could be purified to a degree of homogeneity similar to the wild-type C180 peptide (Figure 3, upper panel). The C180Y98H peptide was found to be in lower abundance in the periplasmic extract than wild-type C180 peptide and the other C180Y98*n* derivatives which allowed for only an enrichment of the C180Y98H peptide from the periplasmic extract (Figure 3, upper panel). The C180Y98*n* peptides reacted with α-C180 peptide IgG by Western blot analysis (Figure 3, lower panel).

Purified C180Y98*n* peptides were tested for the ability to ADP-ribosylate G_t under linear velocity conditions where the activity was proportional to the concentration of each respective C180 peptide. The specific ADP-ribosyltransferase activities of C180Y98A, C180Y98F, and C180Y98H were within 1-fold of the rate of the wild-type C180 peptide (Table 1). These data indicate that the R group of Y98 does not participate in the ADP-ribosylation of G_t by the C180 peptide.

Table 3: Kinetic Constants for Purified C180Y98*n* Peptides in the NAD Glycohydrolase Reaction

	K_m (μM)[a]	k_{cat}[b]	k_{cat}/K_m[c]
	Experiment A		
C180WT	13.1 ± 2.4	7.4 ± 0.4	0.6
C180Y98A	20.1 ± 3.8	12.1 ± 1.4	0.6
	Experiment B		
C180WT	8.5 ± 1.2	1.8 ± 0.1	0.2
C180Y98A	11.2 ± 2.7	3.5 ± 0.3	0.3
C180Y98F	9.8 ± 1.4	4.5 ± 0.2	0.5

[a] Determined by Michaelis–Menten kinetic analysis using Enzfitter software. [b] k_{cat} was reported as moles of nicotinamide released per hour per mole of C180Y98*n* peptide. [c] k_{cat}/K_m was reported as a ratio of the means.

Analysis of an outer membrane fraction of *E. coli* expressing C180Y98A showed no detectable ADP-ribosyltransferase activity (data not shown). Since the C180Y98A peptide was active following its purification from outer membrane suspensions, it appears that upon extraction of the outer membranes in 8 M urea C180Y98A has refolded to a soluble and catalytically active peptide. Addition of outer membrane fraction of *E. coli* expressing C180Y98A to wild-type C180 peptide did not inhibit the activity of the peptide. This indicated that the reactivation of C180Y98A peptide was not due to removal of an inhibitory component of the outer membrane.

Kinetic Constants of Wild-Type C180, C180Y98F, and C180Y98A in the NAD Glycohydrolase Reaction. Analysis of C180Y98A and C180Y98F with respect to the wild-type C180 peptide was extended to include a determination of their kinetic constants in the NAD glycohydrolase reaction. Within an individual experiment, the K_{mNAD} and k_{cat} of C180Y98A and C180Y98F in the NAD glycohydrolase reaction were similar to those for the wild-type C180 peptide (Table 3). These data show that neither the phenol hydroxyl nor the aromatic ring at residue 98 is required for NAD binding and hydrolysis.

DISCUSSION

Our research has focused on defining the functional domains of the S1 subunit of pertussis toxin. Analysis of recombinant derivatives of S1 has allowed the localization of three functional regions within the primary amino acid sequence: region 1 (residues 1 through 180), which comprises the NAD binding domain and NAD hydrolytic machinery; region 2 (residues 180 through 219), which confers efficient ADP-ribosylation of target protein; and region 3 (residues 219 through 235), which is required for the noncovalent binding of S1 to the B oligomer of pertussis toxin.

Several residues within region 1 have been shown to function in the ADP-ribosyltransferase reaction. W26 is required for high-affinity binding to NAD (Cortina, 1991), while E129 appears to function as a catalytic residue (Barbieri, 1990). Several residues between 8 and 13 of S1 have also been shown to be required for the expression of ADP-ribosyltransferase activity, although the functions of these residues have not been determined (Barbieri, 1989; Cieplak, 1989; Locht, 1989). The present study was initiated following molecular modeling of S1 with the crystal structure of exotoxin A (Domenighini, 1991). Several residues of S1 were aligned within the NAD binding domain of the crystal structure of exotoxin A. These studies predicted that Y98 of S1 represented a conserved tyrosine residue among the ADP-ribosylating exotoxins and should reside within the NAD binding domain. The data

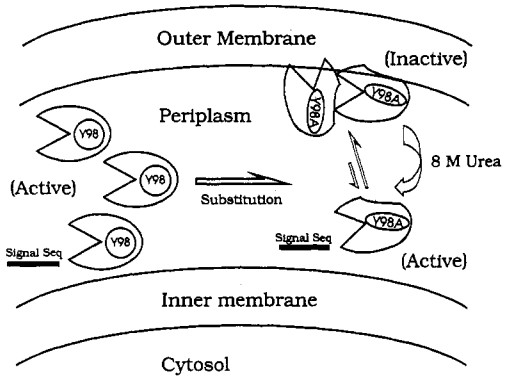

FIGURE 4: Schematic model for the role of residue 98 in the expression of the C180 peptide in *E. coli*.

from our studies indicate that Y98 does not function in the ADP-ribosylation reaction, but rather functions in the folding of S1. This proposed function for Y98 is consistent with an analysis of the effect of substitutions at residue 98 on the predicted secondary structure of S1. In the wild-type sequence of S1, Y98 is predicted to be localized within a turn, while introduction of alanine at residue 98 changes the secondary structure of this region to an α-helix (Xu and Barbieri, unpublished data).

The phenotype of the wild-type C180 peptide and the C180Y98A peptide when expressed in *E. coli* is schematically represented in Figure 4. The aromatic group of Y98 functions to stabilize the conformation of the nascent S1 peptide during cotranslational folding through the bacterial inner membrane such that the peptide folds to a soluble and catalytically active form within the periplasm. Removal of the aromatic R group, represented by C180Y98A, results in the improper folding of the peptide during cotranslational secretion which shifts the equilibrium of the peptide from a soluble and catalytically active form to an improperly folded peptide which is insoluble or localized to the bacterial outer membrane in a catalytically inactive form. Consistent with this model were the observations that (1) the small percentage of C180Y98A that localizes to the periplasmic space is catalytically active, (2) C180Y98A that was isolated from outer membrane preparations had its signal sequence properly cleaved, (3) membranes containing the C180Y98A peptide did not express ADP-ribosyltransferase activity, and (4) following extraction of membranes containing the C180Y98A peptide with 8 M urea a soluble and catalytically active C180Y98A peptide was isolated.

A 10 °C decrease in the cultivation temperature, from 30 °C to 20 °C, had little influence on the expression and subcellular localization of wild-type C180, but reduced the steady-state expression and subcellular distribution of C180Y98A. We interpreted these data to support the model that Y98 was involved in protein folding. In this model, when expressed at 20 °C, C180Y98A was exported through the plasma membrane at a low efficiency, but that which was transported either folded to a soluble form or no longer had a propensity to aggregate or associate with the bacterial outer membrane. Structural components of proteins have been shown to play a role in translocation across the cell membrane, apparently via an interaction with chaperonin proteins (MacIntyre, 1990). Although no consensus sequence has been defined on the secreted protein which is recognized by chaperonins, it is proposed that the secreted protein is recognized by the chaperonin as defined by secondary structure. The chaperonin may maintain the structure of the secreted protein in an export-competent form. The export-incompetent proteins are rapidly degraded in the cytosol. Consistent with this model was the protein structure prediction that the Y98A mutation changed the local secondary structure from a turn to an α-helix. The temperature-sensitive subcellular distribution of C180Y98A suggested that the R group at residue 98 may also play a role in the stabilization of a thermolabile intermediate in the folding pathway of the C180 peptide.

Alignment of the solved crystal structures of DT and exotoxin A places Y65 and Y481 within the cleft of the NAD binding site of their respective toxin (Choe, 1992; Allured, 1987). Although localized within similar regions of their respective toxins, biochemical analysis suggests that Y65 of DT and Y481 of exotoxin A perform different functions in the ADP-ribosylation reaction. Y65 of DT appears to function in the interaction with NAD. Papini et al. showed that Y65 was cross-linked to azidoadenosine and azidoadenine, two photoactivatable analogues of the competitive inhibitors of NAD binding adenosine and adenine, respectively (Papini, 1991). Collier and co-workers showed that Y481 of exotoxin A did not function in NAD binding but rather appeared to influence the binding of the toxin to its target protein EF-2. Kinetic analysis of mutated derivatives of exotoxin A showed that the Y481F mutation had little effect on NAD glycohydrolase activity, but caused a 10-fold reduction in both cytotoxicity and ADP-ribosyltransferase activity (Lukac, 1988). Our data indicate that the S1 homologue of these tyrosine residues, Y98, does not participate in the ADP-ribosyltransferase reaction but appears to function in the folding process of S1. Thus, while Y98 of S1, Y65 of DT, and Y481 of exotoxin A may align as conserved residues among these exotoxins, biochemical analysis showed that these three tyrosines are responsible for different functions within their respective toxins.

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