Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 3075–3079, May 1989
Biochemistry

Identification of amino acid residues essential for the enzymatic activities of pertussis toxin

(site-directed mutagenesis/ADP-ribosyltransferase/bacterial toxins/pertussis vaccines)

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Communicated by Donald R. Helinski, January 23, 1989 (received for review November 8, 1988)

ABSTRACT **The enzymatic ADP-ribosyltransferase activity associated with the S1 subunit of pertussis toxin is considered to be responsible for its biological effects. Although pertussis toxin has no significant homology to other ADP-ribosylating toxins such as diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A, the results presented in this paper show that, as for diphtheria toxin and exotoxin A, tryptophan and glutamic acid residues are essential for the enzymatic activities of pertussis toxin. Moreover, a structural motif can be identified around the critical glutamic acid residue. Chemical modification or site-directed deletion or replacement of Trp-26 abolishes ADP-ribosyltransferase and the associated NAD glycohydrolase activities. Both enzymatic activities are also abolished when Glu-129 is deleted or replaced by aspartic acid. Mutations at the Glu-106 position do not significantly reduce the enzymatic activities of the S1 subunit. The mutations do not affect the ability of the different S1 forms to be recognized by a variety of monoclonal antibodies, including neutralizing antibodies. Pertussis toxin containing a deletion or replacement of Trp-26, Glu-129, or both in the S1 subunit should thus be devoid of toxic activities without losing its reactivity with protective antibodies and, therefore, could be safely included in new generation vaccines against whooping cough.**

Of the various virulence factors synthesized by *Bordetella pertussis*, the causative agent of whooping cough, pertussis toxin (PTX), plays a major role in the pathogenesis of the disease (1). In addition, PTX also protects mice against lethal challenge with virulent *B. pertussis* (2, 3) and constitutes an important component in the new acellular pertussis vaccines (4). On the other hand, active toxin is thought to be associated with the harmful side effects of the current pertussis vaccines (5).

The toxin is a hexameric protein composed of five different subunits, designated S1–S5 according to their decreasing molecular mass (6). The holotoxin is structured in an A–B model, similar to many other bacterial toxins, including cholera toxin (CTX), *Escherichia coli* heat labile toxin (LTX), diphtheria toxin (DTX), *Pseudomonas aeruginosa* exotoxin A (ETX), and many others. The A moiety (S1) contains an ADP-ribosyltransferase activity (7), and the B moiety (S2–S5) contains the target-cell receptor binding sites (8). After binding of PTX to the target cell, the S1 subunit is internalized into the cell, where, upon reduction, it catalyzes the transfer of the ADP-ribose moiety from NAD to the α subunit of different signal-transducing GTP-binding proteins. Depending on the target cell, these GTP-binding proteins may be the inhibitory adenylate cyclase regulatory protein G_i, the retinal signal transducing protein transducin (G_t), the G_o protein with unknown function, or other GTP-binding regulatory proteins (9–11).

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These biochemical modifications result in the numerous toxic activities of PTX, including lymphocytosis, histamine sensitization, potentiation of anaphylaxis, islet activation, and many others (12). The same biochemical mechanisms likely underlie various harmful side effects of the current pertussis vaccines that are probably caused by incomplete or reversible inactivation of the toxin in whole-cell or acellular vaccines. Abolishing through genetic engineering the ability of either the B-oligomer to bind to the target-cell receptors or the A-protomer to carry out the ADP-ribosyltransferase activity might greatly diminish the toxicity of PTX without hampering its immunoprotectivity.

The entire PTX gene has recently been cloned (13, 14) and sequenced (14, 15). To identify potential targets for chemical or genetic manipulation, the primary sequence of the PTX S1 subunit was compared to the amino acid sequences of other bacterial ADP-ribosylating toxins. Except for the A subunits of CTX and LTX, no significant homology could be found (14, 15). The catalytically important residues of these two toxins are not yet known however. DTX and ETX are the best studied toxins with respect to the definition of their catalytic site. In both cases, a glutamic acid residue (Glu-148 in DTX and Glu-553 in ETX) was shown to be located in the nicotinamide subsite (16–19). In DTX, a tryptophan residue, located 5 amino acid residues downstream from the glutamic acid, is also thought to interact with the nicotinamide moiety of NAD (18, 20). The tryptophan residue is conserved 5 amino acid residues downstream of Glu-553 in ETX (18). No such structure could be found in the PTX amino acid sequence. In this report, chemical modification and site-directed mutagenesis were used to show that, nevertheless, a tryptophan residue (Trp-26) and a glutamic acid residue (Glu-129) in S1 are essential amino acid residues for the enzymatic activities of PTX. Furthermore, deletion or substitution of these residues did not abolish the ability of S1 to be recognized by neutralizing antibodies.

MATERIALS AND METHODS

Chemical Modification of Tryptophan Residues. Purified PTX, S1 subunit or recombinant S1 (rS1d), was incubated with a 1000-fold molar excess of 2-hydroxy-5-nitrobenzyl bromide (HNBB) dissolved in dry acetone. The reaction was performed in a 0.1 M sodium acetate buffer (pH 4.0) containing 2 M urea at 25°C for 2 hr. The solution was then dialyzed against 0.1 M sodium phosphate (pH 4.0) for 15 hr at 4°C. In parallel, PTX, S1 or rS1d, was treated the same way, except that HNBB was omitted in the reaction mixture.

Abbreviations: PTX, pertussis toxin; CTX, cholera toxin; DTX, diphtheria toxin; ETX, *Pseudomonas aeruginosa* exotoxin A; LTX, *Escherichia coli* heat labile toxin; HNBB, 2-hydroxy-5-nitrobenzyl bromide; G_i, inhibitory guanine nucleotide-binding regulatory protein; rS1d, recombinant S1 subunit of PTX.

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Recombinant DNA Technology. Standard recombinant DNA techniques were used as described by Maniatis *et al.* (21). The recombinant φRIT20001 was obtained by isolating the 560-base-pair (bp) *Sau*3A fragment containing the coding information for rS1d from pPTX42 (13) and inserting it into the *Bam*HI site of M13mp18 (22). The mutant phages were obtained by site-directed mutagenesis of φRIT20001, using the oligonucleotide-directed *in vitro* mutagenesis system (Amersham) under conditions described by the supplier. The oligonucleotides had the sequences CGTTGTTTCCCGCCGTGAAT (φRIT20002), CGTTGTTTCCGGTCGCCGTGAAT (φRIT20008), TGTCGACGTAGAAGTACGAG (φRIT20006), TGTCGACGTAATCGAAGTACG (φRIT20007), GTGCCAGATAGCTCTGGTAG (φRIT20010), and GTGCCAGATAGTCGCTCTGGTAG (φRIT20011). The DNA sequences were determined by the dideoxynucleotide chain-termination method (23). After mutagenesis, *E. coli* TG1 cells (Amersham) were infected with the phages and grown to 0.3 OD at 37°C in LB medium. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM and incubation continued for 4 hr. Cells were then harvested and analyzed for expression of the different rS1d genes (24).

Other Analytical Procedures. ADP-ribosyltransferase and NAD glycohydrolase activities were assayed as described (24). Purification of PTX and S1 was carried out as described (25), and rS1d was purified as described (C.L., W. Cieplak, C.C., and J. M. Keith, unpublished data). Western blot analyses using monoclonal antibodies were performed as described (24).

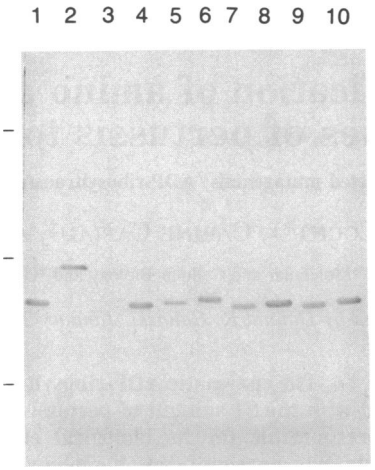

FIG. 2. Western blot analysis of partially purified rS1d, PTX, and cell extracts from *E. coli* infected with recombinant M13 phages. Partially purified rS1d (1 μg, lane 1), PTX (2 μg, lane 2), and total cell lysates of *E. coli* infected with M13mp18 (lane 3), φRIT20001 (lane 4), φRIT20002 (lane 5), φRIT20008 (lane 6), φRIT20006 (lane 7), φRIT20007 (lane 8), φRIT20010 (lane 9), and φRIT20011 (lane 10) were subjected to SDS/polyacrylamide gel electrophoresis and immunoblotting and were incubated with anti-S1 monoclonal antibody B2F8. After derepression of the *lac* promoter for 4 hr cells were harvested and resuspended into 1/10th vol of phosphate-buffered saline; 1/20th vol of 3× Laemmli buffer (29) was added prior to loading onto the gel. The horizontal lines to the left of lane 1 represent the molecular mass markers (43, 25.7, and 14.3 kDa; Bethesda Research Laboratories).

RESULTS

Chemical Modification of Trp-26 in the S1 Subunit. To investigate the importance of tryptophan residues for enzymatic activities of PTX, the tryptophan-modifying chemical reagent HNBB was used. HNBB-treated and untreated toxins were then analyzed for their ability to catalyze the ADP-ribosyltransfer to the 41-kDa G_i protein in Chinese hamster ovary cell membranes. As shown in Fig. 1*A*, untreated toxin and toxin treated with the solvent alone expressed ADP-ribosyltransferase activity. When PTX was treated with HNBB, the ADP-ribosyltransferase activity was much reduced. When the experiment was performed with purified S1 subunit instead of holotoxin, the activity was essentially abolished after treatment with HNBB (data not shown), indicating that the chemical reaction affected an enzymatically important residue in the S1 subunit.

Recent reports have described the synthesis in *E. coli* of individual PTX subunits, including the enzymatically active S1 subunit (24, 26–28). Of the two tryptophan residues in S1, only one (Trp-26) still remains in an enzymatically active truncated version of the S1 subunit synthesized in *E. coli*, designated rS1d (24). Both the specific activity of rS1d and its K_m for NAD were very similar to those of the *B. pertussis* S1 subunit, indicating that all the regions important for enzymatic activity are contained in the truncated rS1d form (C.L., W. Cieplak, C.C., and J. M. Keith, unpublished data).

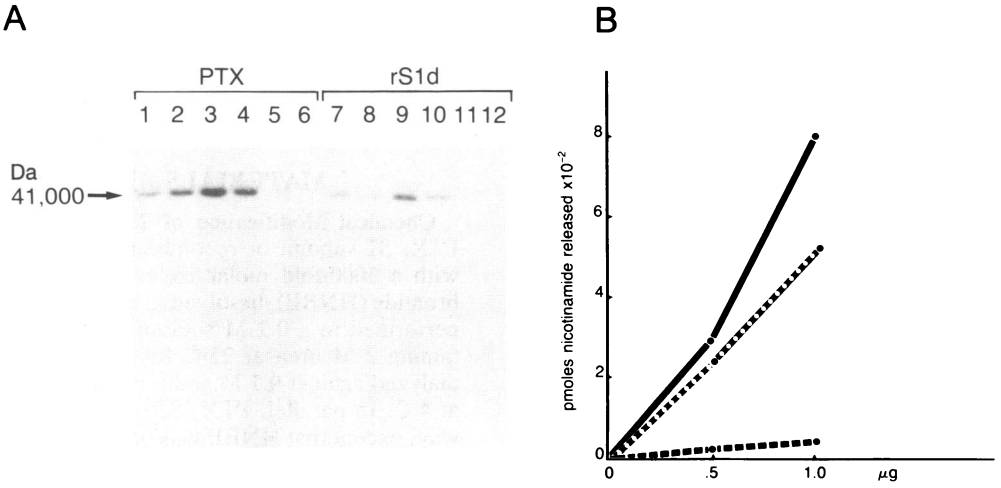

FIG. 1. ADP-ribosyltransferase and NAD glycohydrolase activities of HNBB-treated PTX and rS1d. (*A*) ADP-ribosyltransferase activity of PTX and rS1d. PTX (1 μg, lanes 1, 3, and 5; 0.5 μg, lanes 2, 4, and 6) and rS1d (0.2 μg, lanes 7, 9, and 11; 0.1 μg, lanes 8, 10, and 12) were assayed for their ADP-ribosyltransferase activity after no treatment (lanes 1, 2, 7, and 8), treatment with 5% acetone (lanes 3, 4, 9, and 10), or treatment with HNBB dissolved in acetone (lane 5, 6, 11, and 12). (*B*) NAD glycohydrolase activity of rS1d. rS1d (0.5 μg and 1 μg) was treated with acetone (■●■), HNBB dissolved in acetone (●■■■), or left untreated (●□□□●) prior to NAD glycohydrolase reaction for 45 min.

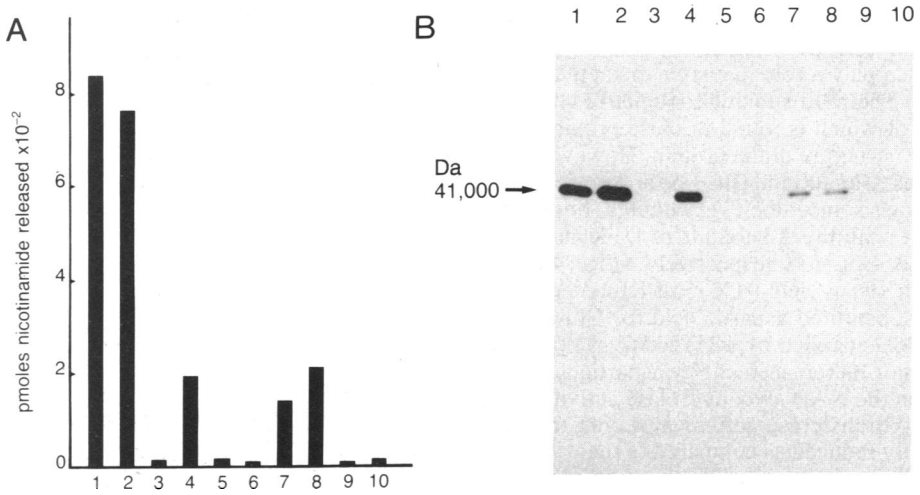

FIG. 3. NAD glycohydrolase and ADP-ribosyltransferase activities of PTX, rS1d, and cell extracts of *E. coli* infected with recombinant phages. NAD glycohydrolase (*A*) and ADP-ribosyltransferase activities (*B*) were measured by using 0.5 μg of partially purified rS1d (lane 1), 2 μg of PTX (lane 2), 25 μl of cell extracts of *E. coli* infected with M13mp18 (lane 3), φRIT20001 (lane 4), φRIT20002 (lane 5), φRIT20008 (lane 6), φRIT20006 (lane 7), φRIT20007 (lane 8), φRIT20010 (lane 9), and φRIT20011 (lane 10). The cell extracts were prepared as described in the legend of Fig. 2, except that after harvesting the cells were resuspended in 1/50th vol of 25 mM Tris·HCl, pH 7.5/25 mM NaCl, lysed in a French pressure cell, and fractionated by centrifugation (24).

Studies on the structure–function relationship of S1 by protein chemical modification and recombinant DNA techniques can thus be greatly facilitated by using the rS1d synthesized in *E. coli*.

Purified rS1d was therefore also subjected to the HNBB treatment and assayed for its ADP-ribosyltransferase and the associated NAD glycohydrolase activities. As shown in Fig. 1*A*, untreated rS1d as well as rS1d treated with the solvent alone were again able to catalyze the ADP-ribosyl transfer to the membrane-bound G_i protein, whereas HNBB-treated rS1d contained no detectable activity. When the NAD glycohydrolase activity was measured in a concentration-dependent fashion, only marginal activity could be detected after HNBB treatment of rS1d, and the activity was reduced to ≈5% when 1 μg of rS1d was assayed (Fig. 1*B*).

Expression of the rS1d Gene by Using the M13mp18 Vector. To facilitate structure–function relationship studies using site-directed mutagenesis, the coding region for rS1d was first subcloned into M13mp18 such that *E. coli* cells infected with the recombinant phage (φRIT20001) synthesized a protein identical to rS1d upon derepression of the *lacZ* promoter. As shown in Fig. 2 φRIT20001-infected *E. coli* cells synthesized a protein that reacted on Western blots with anti-S1 monoclonal antibody B2F8 (30) and comigrated with purified rS1d at ≈22 kDa. *E. coli* cells infected with M13mp18 did not contain such a protein. After lysis of the *E. coli* cells and centrifugation, the supernatant fractions were assayed for the presence of S1-specific NAD glycohydrolase and ADP-ribosyltransferase activities. In the NAD glycohydrolase assay, 0.5 μg of partially purified rS1d and 2 μg of PTX catalyzed the release of, respectively, 825 and 750 pmol of nicotinamide from NAD after 3 hr of reaction. Supernatant fractions of M13mp18-infected *E. coli* cells contained no significant NAD glycohydrolase activity, whereas 25 μl of supernatant fractions of *E. coli* cells infected with φRIT20001 catalyzed the release of 190 pmol of nicotinamide under the same conditions (Fig. 3*A*). When purified rS1d, PTX, and the supernatant fraction of φRIT20001-infected *E. coli* cells were assayed for their ADP-ribosyltransferase activity, specific labeling of the 41-kDa G_i protein was detected. No labeling of this protein was seen when the supernatant fraction of *E. coli* cells infected with M13mp18 was assayed (Fig. 3*B*).

Site-Specific Deletion and Substitution of Trp-26. Single-stranded DNA of phage φRIT20001 was purified and subjected to oligonucleotide-driven site-directed mutagenesis to specifically delete the codon TGG encoding Trp-26 of the rS1d gene. The resulting phages were screened by differential hybridization with the mutagenic oligonucleotide. The accuracy of the mutagenesis was then verified by DNA sequencing of the complete insert.[§] One candidate (φRIT20002) containing the correct mutation was plaque purified and further analyzed for its ability to express the mutated rS1d gene. After induction, *E. coli* cells infected with φRIT20002 were able to synthesize a mutated rS1d form that reacted with monoclonal antibody B2F8 on Western blots (Fig. 2). This rS1d mutant form was designated PTX-rS1d-W26Δ according to the nomenclature of Knowles (31). When extracts of φRIT20002-infected cells were analyzed for their ability to catalyze NAD glycohydrolysis, the activity level was found to be similar to that of extracts of M13mp18-infected cells (Fig. 3*A*). Similarly, no G_i–ADP-ribosyltransferase activity could be detected in extracts of φRIT20002-infected cells (Fig. 3*B*).

In DTX, substitution of Trp-153 by threonine was found to abolish enzymatic activity (18). Therefore, the same tryptophan to threonine substitution was performed at amino acid position 26 in rS1d. *E. coli* cells infected with the resulting mutant phage φRIT20008 were found to synthesize a B2F8-reactive mutant form of rS1d, designated PTX-rS1d-W26T (Fig. 2). When supernatant fractions of φRIT20008-infected cells were assayed for enzymatic activities, no significant NAD glycohydrolase (Fig. 3*A*) or ADP-ribosyltransferase (Fig. 3*B*) activities could be detected.

The combination of site-directed mutagenesis and chemical modification thus revealed that, as for the A subunit of DTX, tryptophan appears to be an important residue for the NAD glycohydrolase and ADP-ribosyltransferase activities of PTX.

Site-Specific Deletion and Substitution of Glu-106 and Glu-129. Since in DTX and ETX, in addition to a tryptophan residue, a glutamic acid residue located 5 residues upstream

[§]It was necessary to sequence the complete rS1d coding regions of all generated mutants, since at low frequency random mutations can be generated in addition to the site-directed mutation. In φRIT20002, such a random mutation did indeed occur. This mutation was fortunately silent and changed the codon AAT to AAC at the Asn-31 position.

also plays an important role in enzymatic activities (16–19), it was of interest to investigate whether any of the glutamic acid residues may also play a role in enzymatic activities of the PTX S1 subunit. The rS1d subunit contains 12 glutamic acid residues, none of which is found in close proximity to Trp-26 in the primary structure of the protein. However, two glutamic acid residues (Glu-106 and Glu-129) are located in an environment that shows some, albeit very limited, homology over a short distance with the A subunits of DTX and ETX around Glu-148 and Glu-553, respectively (Fig. 4). We therefore deleted Glu-106 to yield PTX-rS1d-E106Δ encoded by φRIT20006 and substituted aspartic acid for Glu-106, to yield PTX-rS1d-E106D encoded by φRIT20007. Neither the deletion of Glu-106 nor its replacement by aspartic acid had a significant effect on the NAD glycohydrolase activity (Fig. 3*A*). The ADP-ribosyltransferase activities of both mutants appeared to be slightly reduced as compared to the wild-type activity (Fig. 3*B*). It is of course difficult to compare the specific activities based on reactions performed with crude cell extracts. Interestingly, however, a 4-amino acid insertion 2 residues downstream of Glu-106 in S1 has recently been shown to reduce the ADP-ribosyltransferase activity of PTX. The authors did not measure the NAD glycohydrolase activity of their mutant toxin (12). Since the Glu-106 mutation did not affect NAD glycohydrolysis, it is tempting to speculate that the region around Glu-106 may be involved in the recognition or binding of the acceptor substrate proteins.

We next performed the site-directed mutagenesis to specifically delete the Glu-129 residue and to replace it by aspartic acid and obtained φRIT20010 and φRIT20011 encoding PTX-rS1d-E129Δ and PTX-rS1d-E129D, respectively (Fig. 2). Cell extracts of *E. coli* infected with either mutant phage expressed no detectable NAD glycohydrolase or ADP-ribosyltransferase activities (Fig. 3), indicating that Glu-129 in the S1 subunit of PTX plays an important role in enzymatic activities.

Purification and Estimation of Specific Activities of the Mutated rS1d Forms. Although the mutated rS1d forms were

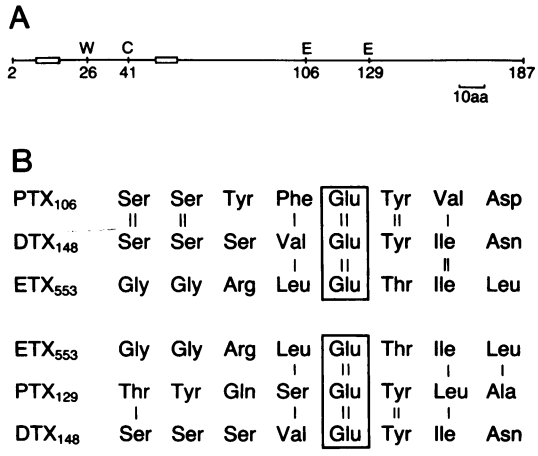

FIG. 4. Structure of rS1d. (*A*) Position of the enzymatically important residues in rS1d. Horizontal line represents the length of the primary sequence (residues 2–187 of S1) of rS1d. Open boxes represent the two regions of S1 that share homology to CTX and LTX. The positions of Trp-26, Cys-41, Glu-106, and Glu-129 are indicated by the vertical lines with single letter codes and position numbers. aa, Amino acid. (*B*) The two regions in the PTX S1 subunit that share limited homology with the regions around Glu-148 of DTX and Glu-553 of ETX. The lined up glutamic acid residues are shown in boxes. Two vertical lines connect strictly homologous amino acids. A single vertical line connects amino acids that share common features (hydrophobicity or size). The PTX sequence is from refs. 14 and 15, the DTX sequence is from ref. 32, and the ETX sequence is from ref. 33.

Table 1. Specific NAD glycohydrolase activities of the rS1d mutant forms

rS1d form	Specific activity	Residual activity, %
rS1d	14.7	100
PTX-rS1d-W26Δ	<0.05	<0.4
PTX-rS1d-W26T	<0.05	<0.4
PTX-rS1d-E106Δ	15.6	106
PTX-rS1d-E106D	17.6	120
PTX-rS1d-E129Δ	<0.05	<0.4
PTX-rS1d-E129D	<0.05	<0.4

Specific activity is expressed as pmol of nicotinamide released per μg of the rS1d polypeptide per min of reaction.

synthesized in *E. coli* at levels roughly similar to the wild-type form in φRIT20001-infected cells, as judged by visual inspection of the Western blot shown in Fig. 2, it was difficult to directly compare the specific activities of the mutant forms with the wild-type form because of the presence of a NAD glycohydrolase inhibitor in crude *E. coli* cell extracts (W. Cieplak and C.L., unpublished data). Therefore, the different mutant subunit forms and the nonmutated rS1d were partially purified by DEAE-cellulose chromatography. The concentration of the purified polypeptides was estimated by comparative slot blot analysis using the B2F8 monoclonal antibody. From 10 to 500 ng of each rS1d form was then tested in the NAD glycohydrolase assay and the specific activities of the mutant forms were compared to the wild-type rS1d. As shown in Table 1, PTX-rS1d-W26Δ, PTX-rS1d-W26T, PTX-rS1d-E129Δ, and PTX-rS1d-E129D all expressed <0.05 pmol/min per μg of NAD glycohydrolase activity, reaching the detection limit of the assay. When the specific activities were compared to the nonmutated rS1d specific activity, the four mutants were found to express <0.4% of the wild-type activity. On the other hand, both PTX-rS1d-E106 and PTX-rS1d-E106D expressed high NAD glycohydrolase activities (15.6 and 17.6 pmol·min^{-1}·μg^{-1}, respectively), similar to the nonmutated rS1d activity (106% and 120%, respectively).

Antigenicity of the Mutated rS1d Forms. PTX, the partially purified rS1d, and the different mutant forms were then analyzed for the presence of important epitopes. Polyclonal anti-PTX antiserum, as well as over 20 different monoclonal antibodies, including antibodies that neutralize PTX activities, reacted with the S1 subunit of PTX, rS1d, as well as all mutated forms of rS1d. The epitopes for neutralizing monoclonal antibodies have recently been shown to be conformational epitopes (34), although the antibodies reacted with S1 on Western blots. The conservation of the conformational epitopes of the rS1d mutant forms indicates that the three-dimensional structure of rS1d is not grossly affected by the different mutations and, thus, that Trp-26 and Glu-129 are catalytically, rather than structurally, important residues.

DISCUSSION

Using chemical modification and site-directed mutagenesis techniques, we have identified two amino acid residues, Trp-26 and Glu-129, that are essential for the enzymatic activities of PTX. The specific deletions or substitutions of these residues did not profoundly alter the tertiary structure of the S1 subunit, since all the mutants were recognized by polyclonal and a number of monoclonal antibodies, some of which are PTX neutralizing and known to recognize conformational epitopes.

Since both NAD glycohydrolase and ADP-ribosyltransferase activities were affected by the mutations at either position 26 or 129, we conclude that these alterations interfere with binding of NAD and/or catalysis of the glycohydrolysis, rather than affecting only recognition of, or binding

to, the acceptor substrate G_i protein. Whether the mutations of Trp-26 and Glu-129 have the same properties on the full-length S1 subunit or the holotoxin is not known. Trp-26 lies between two regions that show strong amino acid homology to the A subunits of CTX and LTX (see Fig. 4*A*). However, no tryptophan residue can be found between the two boxes in CTX or LTX. Despite the evolutionary divergence and the absence of significant amino acid sequence homology between DTX, ETX, and PTX, it nevertheless appears that the enzymatically important amino acid residues—i.e., tryptophan and glutamic acid—are identical in all three toxins, suggesting a similar enzymatic mechanism of these ADP-ribosylating toxins. Because of the covalent attachment of the nicotinamide moiety via its number 6 carbon to the decarboxymethylated γ-methylene carbon of Glu-148 after photoaffinity labeling of the A subunit of DTX with NAD (35), it was concluded that the carbon 6 atom of the nicotinamide ring is in close proximity to the γ-methylene group of Glu-148 in the DTX–NAD complex. ETX is also efficiently photolabeled at the Glu-553 position (18). On the other hand, in DTX the nicotinamide ring also interacts with Trp-153 (36), and chemical modification (20), as well as replacement of that residue by threonine (18), abolished enzymatic activity. Whereas in DTX and ETX the important glutamic acid and tryptophan residues are separated by only 5 amino acid residues, the important residues are located in distant positions on the primary structure of PTX. Protein folding must therefore bring Trp-26 in close proximity to Glu-129, such that both residues can interact with the nicotinamide ring of NAD.

As exemplified by this study, to identify functionally important residues, it may be more profitable to search primary structures for motifs of similarity to other functionally related proteins, rather than to search for significant stretches of homology between the proteins. Although no significant homology was found between PTX, DTX, and ETX (14, 15), a functional motif around the critical glutamic acid residues could be identified (Fig. 4). Identification of such motifs in conjunction with protein chemical and site-directed mutagenesis techniques may generally be a useful alternative to homology searches for the identification of functionally important residues in enzymes.

Because of the dramatically reduced enzymatic activities of the described PTX mutants and the conservation of their conformational toxin-neutralizing epitopes, they have a good potential to be developed as an essential compound of a safe nontoxic pertussis vaccine. Alternatively, by introducing the mutations into the *B. pertussis* genome through allelic exchange with the wild-type PTX operon, it may be possible to obtain a genetically detoxified holotoxin suitable for vaccine development. The methodology for such an allelic exchange has already been proven successful (12). PTX, as well as other antigens, such as the filamentous hemagglutinin, can then be purified from these strains and used in a new generation component vaccine, without the need for chemical detoxification. As at least some of the side effects associated with the current pertussis vaccines can be attributed to reversion of the chemically inactivated PTX, a vaccine based on genetically inactivated toxin should be a safer alternative.

Note Added in Proof. After this manuscript was submitted, Pizza *et al.* (37) showed that a Glu-129 to Asp mutant form of full-length recombinant S1 expressed <5% NAD glycohydrolase activity.

We thank Drs. J. M. Keith, J. G. Kenimer, and M. Francotte for the monoclonal antibodies; E. Simoen for polyclonal antiserum; Dr. B. Colau for synthesizing the oligonucleotides; Drs. J. J. Mekalanos and T. Cabezon for fruitful discussions; Drs. M. De Wilde, J. Cohen, and N. Harford for critically reading the manuscript; D. Plainchamp for excellent technical assistance; and H. Monnig and C. Van Roy for preparing the manuscript. This work was supported by the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (Convention 4726).

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