NOTES

Effects of Mutations on Enzyme Activity and Immunoreactivity of the S1 Subunit of Pertussis Toxin

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By introducing a series of six different substitutions at and around position 9, we investigated the structural requirements of the amino-terminal region of the S1 subunit of pertussis toxin for both enzyme activity and immunoreactivity. All mutant S1 analogs with a substitution at this location exhibited severely decreased ADP-ribosyltransferase activity (range, 400- to 2,500-fold). In contrast, alteration of arginine 58 had considerably less effect. The reactivity of the mutant molecules with monoclonal antibody 1B7 varied with the nature of the substitution. These findings indicate an absolute requirement for the presence of an arginine residue at position 9 for the maintenance of efficient ADP-ribosyltransferase activity and illustrate the specific participation of vicinal residues in the formation of the protective epitope.

Pertussis toxin (PTX) is a hexameric protein produced by Bordetella pertussis that is composed of five different subunits, termed S1 through S5 (13). PTX exerts a diverse array of biological effects in vitro and in vivo and is believed to occupy a central role in the pathogenesis of whooping cough, or pertussis (15). Many of these effects appear to result from the ability of the S1 subunit of PTX to catalyze the ADP-ribosylation of guanine nucleotide-binding proteins of the adenylate cyclase complex (6), with NAD+ as the ADP-ribose donor.

We have previously reported that deletion of a small amino-terminal region of the S1 subunit, delineated by amino acid residues 8 to 15, reduces drastically the ADP-ribosyltransferase activity and results in the loss of a protective epitope (5). This region is one of two areas of the S1 subunit that exhibits amino-acid-sequence homology to the active A fragments of two other ADP-ribosylating toxins, cholera toxin and Escherichia coli heat-labile toxin 1 (9). More recent analyses showed that replacement of an arginine residue at position 9 with lysine decreased ADP-ribosyltransferase activity by a factor of several thousand, while other single isocionic substitutions at positions 8, 11, 12, and 13 reduced the enzymatic activity of S1 to a lesser extent or not at all (4). All of these changes, including Arg-9→Lys, resulted in the retention of a protective antigenic determinant. This determinant has been shown to be a complex epitope that is composed of at least two discrete regions (2); the substitutions therefore did not appear to impart gross structural alterations to the molecule. Although these results suggested the importance of the presence of an arginine at position 9 for the ADP-ribosyltransferase activity of S1, they did not eliminate the possibility that the loss of activity was peculiar to the Arg→Lys substitution. Therefore, we have investigated more systematically the influence of several different types of substitutions at position 9 and in its immediate vicinity on the enzymatic activity and the immunoreactivity of S1.

Mutagenesis and expression of S1 subunits. Using oligonucleotide-directed mutagenesis (7, 14), we introduced seven changes into the N-terminal region of S1 (Fig. 1). Five of these changes were at position 9. The mutant genes carried by plasmid pTXS13 (8) were expressed in E. coli JM101, and after the cells were disrupted, the resultant proteins were extracted with 8 M urea and dialyzed (8). Since the S1 analogs were expressed at a level that did not allow unambiguous visualization on stained polyacrylamide gels, the amount of recombinant protein produced was measured with immunoblots (3) probed with a mouse anti-S1 polyclonal antibody (aTr14) that is known to react uniformly with various N-truncated forms of the protein (5). The relative amount of S1 analog present in each extract was estimated by densitometric scanning of the immunoblots. Table 1 summarizes the results of the quantitative immunoblot analyses.

ADP-ribosyltransferase activity of the mutant S1 subunits. After the extracts were appropriately diluted to standardize the amount of immunoreactive material, they were assayed for their ADP-ribosyltransferase activities with purified transducin as the acceptor substrate (5). Background activity was measured by using an extract prepared from JM101 cells which contained pPTXR, a pUC18-based plasmid in which another part of the PTX operon was inserted in the reverse orientation. Assays were performed with several concentrations of each preparation. The results of several such experiments show that the activities of the position 8 and 9 mutants are reduced by factors ranging from 400 to 2,500 (Table 1).

These results extend our previous observations and indicate that the reduction in enzyme activity incurred by alterations of Arg-9 is not unique to the replacement of arginine with lysine. Interestingly, the S13R9H mutant analog exhibited reproducibly higher activity than the other position 9 mutants; this result may reflect a more conservative change in the steric configuration of the side chain than

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in that of the other mutants. This observation, together with the observed effect of the R9K substitution, suggests that the size and configuration of the side chain of the residue at position 9 are critical for the enzymatic activity of S1. The large effect resulting from substituting tyrosine for an aspartate residue at position 8 indicates that the presence of a local net positive charge in this area may also be important to the retention of enzymatic activity.

In addition to residues 8 to 15, the sequence of 8 amino acids located between positions S1 and S8 in S1 exhibited significant homology with an analogously located sequence in choler toxin and E. coli heat-labile toxin I (Fig. 1). To investigate the possibility of the involvement of this region in the enzymatic activity of S1, we mutagenized the only charged amino acid of this region that is conserved in cholera toxin and E. coli heat-labile toxin I. The R58K substitution decreased the ADP-ribosyltransferase activity of the S1 subunit by only severalfold and minimally affected reactivity with IB7. This result, in contrast to those of Pizzia et al. (11), who employed a mutant double substituted in this region, suggests that this area of the molecule may not be directly involved in the catalytic process but may be important for correct folding of the protein.

**Immunoreactivity of the mutagenized S1 subunits.** Table 1 also summarizes the results of the quantitative scanning densitometric analyses of blots in which the reactivities of the mutants with three different antibodies were determined relative to the reactivity of the parent molecule. These three antibodies (monoclonal antibodies IB7 and B2F8 and polyclonal antibody RoPTX) were obtained from animals immunized with whole PTX and are known to require the first region of homology to display immunoreactivity in immunoblots (5, 11). A comparison of the results with αTr14 and the results with these three antibodies shows that the later exhibited variable reactivity with the different S1 analogs that contain amino acid substitutions or deletions at position 8 or 9. However, the three antibodies exhibited patterns of reactivity similar to those of the mutant molecules. For example, increasing steric or ionic alteration at position 9 affected the reactivities of all three antibodies in a similar fashion (i.e., S13 ≈ S13R9K > S13R9H > S13R9A > S13R9A). The results obtained with mutant S13R9A indicate that the presence of a positive charge is not absolutely required to allow these antibodies to recognize S1. However, the presence of a negative charge or the absence of the polar character of the residue in position 8 (Y8D) seems to dramatically decrease the immunoreactivity of S1 with these antibodies. Finally, results with the two deletion mutants S13R9A and S13Y8D suggest that the size of this region is also critical in maintaining the integrity of the epitope recognized by IB7, B2F8, and RoPTX.

Unlike previous workers (1, 11), we detected enzymatic activity even with our least active mutant. This finding stresses the importance of the sensitivity of the enzymatic assay to be used in the evaluation of S1 analogs potentially suitable for an acellular vaccine. Furthermore, as the presence of even trace amounts of active (nondetoxified) PTX in the current whole-cell vaccine has been proposed to be responsible for the severe side effects observed at low frequency in the vaccinated population (10, 12), caution must be exercised in the extrapolation of results obtained from an enzymatic assay to in vivo toxicity. Even such a molecule as S13R9K, which exhibits a reduction in activity of more than 3 orders of magnitude, might not be suitable for an acellular vaccine; the true effect of such a mutation will have to be estimated by using a mutant holotoxin in vitro and in vivo assays. Attempts should also be made to further lower the activity of S1 by evaluating the effects of mutations at multiple locations on enzyme activity and immunogenicity.

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**LITERATURE CITED**


