Binding to sulfatide and enterotoxicity of various *Escherichia coli* STb mutants

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Binding of the 48 amino acid polypeptide of the mature heat-stable *Escherichia coli* enterotoxin b (STb) to the functional receptor sulfatide (SFT) constitutes the first step in inducing secretory diarrhoea in the intestinal lumen of animals. The NMR structure of this toxin dictated the choice of amino acids for site-directed mutagenesis to delineate the binding site of STb to SFT. Amino acids facing the solvent either in the loop or the hydrophobic α -helix were selected. Seventeen site-specific mutants of STb toxin were produced and purified by high-pressure liquid chromatography. Enterotoxicity of the 17 mutants was determined using a rat loop assay and binding was evaluated using a microtitre plate binding assay. Both hydrophobic and electrostatic interactions are important for STb attachment. When mutations (F37K, I41S and M42S) were introduced into the hydrophobic α -helix to lessen hydrophobicity, binding activity and enterotoxicity decreased by more than sixfold. The loop defined by C21 and C36 also made specific contributions. Mutants generated at basic residues (K22, K23 and R29) within this region exhibited both reduced binding activities and reduced toxic activities. For all STb mutants constructed and analysed, when binding to SFT was reduced, a reduction in toxicity equivalent or greater was noted, indicating that binding to SFT is a step that precedes the toxic effect observed for STb toxin. Significantly, when the negatively charged D30 was substituted for either alanine or valine, the binding to SFT was about twice that of native STb, whereas the enterotoxicity was reduced by half.

Keywords: enterotoxin b, mutagenesis, binding, ETEC

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) strains cause severe diarrhoeal diseases in humans and animals. Two classes of enterotoxins are responsible for the pathogenesis: heat-labile enterotoxins (LTI and LTII), which are structurally and functionally related to the cholera toxin (Kaper *et al.*, 1995; Spangler, 1992), and the heat-stable toxins (STs). Two subtypes, sharing no homology at the nucleotide or amino acid levels, are identified as heat-stable toxins, namely STa and STb (Dubreuil, 1997). These low-molecular-mass toxins are extracellular enterotoxins; STb is methanol-insoluble whereas STa is not. STa is an 18 or 19 aa acidic peptide that binds to particulate transmembrane guanylate cyclase C (GC-C). This binding leads to an increase in cGMP and eventually the opening of the cystic fibrosis

transmembrane regulator (CFTR) channel responsible for chloride secretion (Goldstein *et al.*, 1994).

In contrast to LT and STa toxins, there is no evidence that STb is associated with an intracellular elevation of cyclic nucleotide (Kennedy *et al.*, 1984; Peterson & Whipp, 1995; Weikel & Guerrant, 1985). Instead, *in vitro* studies have shown that the intestinal secretion pathway involves the activation of a GTP-binding regulatory protein ($G_{\alpha 13}$ protein) sensitive to pertussis toxin. This action results in an intracellular calcium elevation through a receptor-dependent ligand-gated Ca^{2+} channel (Dreyfus *et al.*, 1993). The ensuing high level of intracellular Ca^{2+} is presumably involved in activation of a calmodulin-dependent protein kinase II (CaMK II) which could open an undetermined ionic channel (Fujii *et al.*, 1997). In addition, intracellular concentration of Ca^{2+} may regulate phospholipases A₂ and C, which catalyse the release of arachidonic acid from the membrane phospholipids, and ultimately the

Abbreviations: CD, circular dichroism; SFT, sulfatide.

Fig. 1. Amino acid sequence of mature STb enterotoxin. C10 to K23 represents the stretch forming the amphipathic α -helix and G38 to A44 represents the hydrophobic α -helix. Bold letters represent amino acids targeted for mutagenesis.

Table 1. Biological activity of STb mutants as determined in previous studies

Mutations	Biological activity (%)	Reference
STb	100.0	
K22L	10.0	Fujii <i>et al.</i> (1994)
K23T	10.0	Fujii <i>et al.</i> (1994)
K22I, K23N	2.5	Fujii <i>et al.</i> (1994)
R29I	50.0	Fujii <i>et al.</i> (1994)
R29S	12.7	Dreyfus <i>et al.</i> (1992)
D30V	39.0	Dreyfus <i>et al.</i> (1992)

formation of prostaglandin E₂ (PGE₂) known as a secretory agent (Fujii *et al.*, 1995; Hitotsubashi *et al.*, 1992; Peterson & Whipp, 1995). Recently, our laboratory demonstrated *in vivo* the functionality of sulfatide (SFT) as a receptor for STb (Rousset *et al.*, 1998). We determined its affinity constant (K_d) between 2 and 6 μ M (Beausoleil & Dubreuil, 2001). Unsolved remain the steps between the attachment of STb to its receptor and the activation of the G protein presumably involved in signal transduction.

STb toxin synthesized as a 71 aa precursor (Fig. 1) undergoes cleavage of the N-terminus signal sequence (residues 1–23) (Kupersztoch *et al.*, 1990). This process results in a 48 aa polypeptide of 5200 Da, pI 9.6 (Handl *et al.*, 1993). Maturation also involves the formation of two disulfide bridges: between C10 and C48 and between C21 and C36. Both disulfide bridges are important for stabilization of the molecule's tertiary structure (Sukumar *et al.*, 1995) and are indispensable for enterotoxicity (Arriaga *et al.*, 1995; Okamoto *et al.*, 1995). NMR studies of STb have established a structure having two antiparallel α -helix motifs separated by a loop. An amphipathic helix between residues 10 and 22 exposes several polar side chains to the solvent. Facing this is a hydrophobic helix extending from residue 38 to 44. The glycine-rich loop presents some amino acids that

previous mutagenesis studies have demonstrated as being important in enterotoxicity (Sukumar *et al.*, 1995).

Mutagenesis studies (Table 1) (Dreyfus *et al.*, 1992; Fujii *et al.*, 1994) have reported the relative importance of some amino acids in enterotoxicity, but none have been conducted in order to evaluate their implication in the attachment to the SFT receptor. Consequently, nothing is known about the possible segregation between the binding domain and the domain responsible for enterotoxicity. Since it has been shown that the glycine-rich motif could participate in various functions in proteins, such as in the flexible arm of the monomeric α -haemolysin of *Staphylococcus aureus* allowing its membrane insertion (Ward *et al.*, 1994), it was of interest to further investigate the glycine-rich loop of STb between C21 and C36. Likewise, the hydrophobic region of the STb α -helix shares some structural properties with the *S. aureus* pore-forming α -toxin, which also possesses amphipathic and hydrophobic α -helices involved in pore formation (Lala & Raja, 1995; Ward *et al.*, 1994).

Although a small polypeptide, STb has primary and secondary structures possessing some features that could play important roles in binding to a receptor and either cell penetration or signal transduction involving a G protein. Our objective was to confirm or refute the role of selected amino acids in enterotoxicity and also to delineate the amino acids involved in the interaction of STb with SFT.

METHODS

Oligonucleotide-directed mutagenesis and DNA sequencing.

Oligonucleotide-directed site-specific mutagenesis was performed using the USE Mutagenesis Kit purchased from Pharmacia Biotech. The mutagenic primers (Table 2) used to generate point mutations were synthesized by Gibco-BRL. The plasmid pMal-STb, already described (Bossé *et al.*, 1993), served as template for simple and double mutations. The mutated plasmid pMal-STb-R29A served for one double (K23A-R29A) mutation. All mutants were confirmed by automated sequencing with the *RmaII* primer 5'-GTAACG-CCAGGGTTTCC-3' at the DNA sequencing core facility (University of Maine, Orono, USA) by using an ABI model 373A stretch DNA sequencer.

Purification. To obtain pure STb mutants and native STb enterotoxin, the peptides were produced according to a previously described method (Handl *et al.*, 1993). Briefly, *E. coli* strain HB101, harbouring the plasmid pMal-STb with or without a selected mutation, highly expressed the fusion protein, MBP-STb or MBP-mutated STb, under the control of P_{tac} . Following the recovery from osmotic shock, the fusion protein was affinity purified by using an amylose resin (New England Biolabs) and cleaved with the protease factor Xa. The cleaved material was loaded on a C₈ reverse-phase microbore column (Applied Biosystems) and eluted with an acetonitrile gradient (model 130A Separation System, Applied Biosystems). Purified peptides were lyophilized and stored at –20 °C. The purity of the toxin and mutants was routinely verified by N-terminal sequence analysis using Edman degradation (Applied Biosystems model 470A gas-phase sequencer) as previously described (Handl *et al.*, 1993).

Circular dichroism analysis. Circular dichroism (CD) measurements were carried out on a Jasco J-710 spectropolarimeter.

Table 2. Synthetic mutagenic primers used in this study

Mutations	Oligonucleotide sequence*
K22 → A	CCCTAAAAAACCTTT <u>CGC</u> ACAACCTTTCCTTGGC
K23 → A	CCCCTAAAAAACCTG <u>CCTT</u> ACAACCTTTC
K22 → A, K23 → A	CCCCTAAAAAACCTG <u>CCG</u> ACAACCTTTCCTTGGC
G24 → A	CCCCTAAAAACGCTTTCTTACAACCTTTC
G27 → P	CCATCTCTAACC <u>G</u> GTAACCAACC
R29 → A	GCAGTACCATCTGCAACCCCTAAAAAAC
K23 → A, R29 → A	CCCCTAAAAAACCTG <u>CCTT</u> ACAACCTTTC
	GCAGTACCATCTGCAACCCCTAAAAAAC
D30 → A	CCAGCAGTACCAGCTCTAACCCC
D30 → V	CCAGCAGTACCA <u>ACT</u> CTAACCCC
G31 → A	GCTCCAGCAGT <u>AG</u> CATCTCTAACCC
G34 → A	CCAAAGCATGCTG <u>C</u> AGCAGTACC
F37 → K	CCATTATTTGGGCGCCTTT <u>AC</u> ATGCTCCAGC
F37 → D	CCATTATTTGGGCGC <u>ATCA</u> CATGCTCCAGC
I-41 → S	GCTGCAACCATCGATTGGGCGCCAAAGC
M42 → S	CCTTTTGCTGCAACGCTTATTTGGGCGCC
M42 → R	CCTTTTGCTGCAACGCGTATTTGGGCGCC
I41 → E, M42 → R	CCTTTTGCTGCTGCAACGCGT <u>TC</u> TGGGCGCCAAAGC

* The changed bases on the antisense strand of the plasmid Pmal-STb are underlined.

Far-UV (300–185 nm) runs were conducted in a 0.1 cm cell maintained at room temperature. Concentrations of STb and mutant STb were adjusted to 0.75 mg ml⁻¹ in phosphate buffer 0.02 M (pH 7.2). The data collected were computer-averaged for ten scans, baseline subtracted, and smoothed.

Microtitre plate binding assay. U-bottom polystyrene microtitre plates (Serocluster, Costar) were coated by overnight evaporation of 50 µl methanol-diluted SFT (500 ng) (Sigma). Free sites were blocked with 200 µl per well phosphate-buffer (PBS)-casein (0.3 %, w/v) pH 7.4 for 2 h at room temperature. Purified STb toxin (500 ng) or mutant STb in 50 µl PBS (0.1 M phosphate containing 150 mM NaCl) pH 7.0 were added per well in triplicate for 1 h at 37 °C. After three washes with PBS-casein, each well was filled with 50 µl rabbit monospecific anti-STb antibodies diluted 1:1000 in PBS-casein for 1 h at 37 °C. The plates were washed three times with PBS-casein. Goat anti-rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch) was diluted 1:3000 in PBS-casein and 50 µl was applied to each well before incubation at 37 °C for 1 h. Following three washes with PBS-casein, 50 µl chromogenic substrate ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] (Sigma) (0.4 mM) dissolved in citrate buffer pH 4.0 containing 0.5 mM H₂O₂ was added. The absorbance was measured at 410 nm using an automatic ELISA reader (model MR5000, Dynatech Laboratories).

Triplicates of methanol with and without SFT served as negative controls to evaluate the background after the blocking step and the reaction of anti-STb antiserum with SFT, respectively. For analysis, the mean absorbance obtained for these wells was subtracted from the mean absorbance for STb or each mutant tested. The value for STb was considered as 100 % binding and all the data were calculated relative to this value. The final value represents the mean of at least two assays.

Ligated rat jejunal loop assay. Enterotoxicity studies were conducted using a ligated rat jejunal loop assay as described previously (Dubreuil *et al.*, 1991). Briefly, 6- to 8-week-old white male rats (Sprague Dawley CD; Charles River Ltd) were

fasted for 48 h and anaesthetized. The small intestine was exteriorized following a midline abdominal incision and rinsed for 5 min with 8 ml 0.85 % saline solution containing 300 µg soybean trypsin inhibitor (Boehringer Mannheim) ml⁻¹. A series of seven 5-cm-long ligated segments (loops) was made in the jejunum. The posterior 30 % of the small intestine starting at the caecum was not used. Each loop received 5 µg test material in 500 µl 20 mM Tris/HCl buffer (pH 6.8) containing 300 µg trypsin inhibitor ml⁻¹. Loops receiving only the buffer were considered as the negative controls. The abdominal incision was closed. Three hours later, the rats were killed and the amount of fluid in each loop was measured. Results are expressed as the ratio of millilitres fluid accumulated per centimetre ligated jejunal segment. Each mutant was tested twice in each rat, varying the position among the loops, and at least six rats were used for each mutant. To minimize the effect of sample position, two mutants and two STb-positive controls were alternated in each rat. Samples were always separated by buffer (negative control).

For analysis, the mean of three negative controls was subtracted from the values of three positive controls (STb alone) and from two samples of each mutant tested, in the same rat. Finally, from these results the mean and standard deviation were calculated, omitting the highest and the lowest values. The value for STb was considered as 100 % biological activity and all the data were transformed relative to this value.

RESULTS

Rationale for production of mutant STb molecules

To determine which STb mutants would contribute most to the understanding of the steps involved in the mechanism of action of this toxin, we first indexed all mutants produced in previous studies (Table 1). Those mutants were tested for biological activity, as indicated in Table 1, but were not assayed for binding to SFT. The

Fig. 2. HPLC chromatograms at 220 nm of purified STb and selected mutants. **A**, mutant I41E-M42R, representing the group losing hydrophobicity upon mutation; **B**, native STb; **C**, mutant K22A-K23A, representing the group acquiring hydrophobicity upon mutation. Polypeptides were eluted with a gradient of acetonitrile containing 0.1% trifluoroacetic acid using a reverse-phase C_8 microbore column.

sequence of STb (Fig. 1) and the available NMR model of STb (Sukumar *et al.*, 1995) allowed us to more precisely target the amino acids facing the solvent at relevant positions and/or properties that could yield information on whether or not the binding and toxicity events are associated. On the basis of the information available and the aim of the study, 17 mutants were constructed and the mutagenic primers used in this study are shown in Table 2.

Analysis of STb mutants by CD

Properly oxidized disulfide bonds have been shown to be important for full STb enterotoxicity (Arriaga *et al.*, 1995). In our study, we observed that STb attachment to SFT is also dependent on the presence of oxidized disulfide bonds. Upon β -mercaptoethanol treatment, the binding to SFT decreased to 10% (data not shown). Because important conformational changes induced by point mutations in STb could prevent the proper oxidation of cysteines in disulfide bridges, we analysed each mutant by measuring their CD spectra to confirm that all mutants retained their full structural integrity and in particular the presence of both disulfide bonds. Thus, the main chain structure of the STb enterotoxin, representing the α -helix-loop- α -helix pattern, appears not likely to be greatly affected by our site-directed mutagenesis. The CD spectra were similar to native STb, with the exception of mutant M42S, which seems slightly different (data not shown).

Hydrophobicity profiles of STb mutants

As shown in Fig. 2, STb mutants were purified to homogeneity as demonstrated by the single elution peak using a C_8 reverse-phase column. These chromatograms represent the hydrophobicity profiles of the toxin mutants generated. As expected, mutations changing a non-polar residue for a polar one in the hydrophobic α -helix (F37, I41 and M42) diminished their retention time

(t_R) compared to native STb toxin. Almost all toxin mutants in the loop (C21–C36) were produced using the alanine-scanning procedure (Cunningham & Wells, 1989). In this case, the t_R of the mutants was increased, indicating a predicted loss of polarity.

Relative importance of the STb loop in attachment to SFT

The microtitre plate binding assay, a technique derived from ELISA, allows a useful quantification of the affinity between a ligand and a receptor. In our study, we measured the interaction of different STb mutants with their known SFT receptor. This glycosphingolipid is a sulfated galactose residue joined to a ceramide moiety [Gal(3SO₄) β 1Cer]. The evaluation of binding was done using the optimal parameters as determined for wild-type STb toxin. All mutants and STb toxin (1 μ g) reacted equally with the polyclonal monospecific anti-STb serum (1/300) when blotted on Immodyne membrane (Pall Biosupport Division). Quantitative binding analysis was determined by densitometric analysis as already described (Rousset *et al.*, 1998) (data not shown). We first examined the charged amino acids present in the loop of STb. As shown in Fig. 3, a double mutation introduced in the contiguous lysines (K22–K23) gave an average binding diminution of 91%. When these lysines were mutated independently, relative attachment was respectively 52% and 54% of native STb. Arginine R29 was also changed for alanine, alone or co-mutated with K23. These modified toxins exhibited SFT-binding levels of 45% and 40%, respectively. The double mutation K23A-R29A, however, did not show any decrease in SFT-binding affinity over that obtained with the single mutations K23A or R29A.

To establish the implication of the loop flexibility or simply the role of glycines in SFT binding, all glycines present in the loop (G24, -27 and -31) were subjected to site-directed mutagenesis. When the resulting mutant toxins were tested for their binding relative to the wild-type, values were reduced to 49, 29 and 41%, whereas for G34 the binding value was 118%. Aspartate D30, also present in the loop, was changed for either alanine or valine, resulting in hyperattachment of STb polypeptide to SFT. The toxin with the D30A mutation bound less to SFT (150%) than that with the D30V mutation (210%).

Importance of the hydrophobic α -helix in attachment to SFT

To reduce the hydrophobicity features of the C-terminal α -helix, some highly non-polar residues pointing to the solvent (F37, I41 and M42) were targeted. None of them, even when mutated in combination, changed for negatively charged, positively charged or polar residues, showed a binding activity greater than 10% of native STb, with the exception of mutant M42R, which expressed the same attachment to SFT as STb. Among all mutants, the double mutation I41E-M42R displayed the lowest affinity for the receptor (3%).

Fig. 3. Relative binding to SFT (grey columns) and enterotoxigenicity (white columns) of various STb mutants compared to STb native toxin. The left panel shows the results obtained for substitutions in the loop, the right panel, substitutions introduced in the C-terminal hydrophobic α -helix. STb binding to SFT and enterotoxigenicity was taken as 100% and the relative activity of the mutants calculated as percentage. The binding activity of mutants over 100% are indicated as numbers at the top of the respective column. No experiments to determine the biological activity of mutants D30V and F37D were done. The standard deviations are shown. Mutants significantly different from STb were determined using Student's *t*-test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Relative biological activity

The rat loop assay is a standard method that has proven to be useful for evaluating the toxicity of STb toxin. Among the 12 single mutants depicted in Fig. 3, the greatest decreases in toxicity were noted with three residues (F37K, I41S and M42R) located in the non-polar helix and two located in the loop (G24A and G31A). The biological activity detected in rat loop assay for these five mutants averaged 11, 11, 6, 11 and 1%, respectively. For the double mutation I41E-M42R, intestinal enterotoxigenicity (11%) was similar to the single mutations. Inside the STb loop, diminution of the toxicity was observed with three single mutants assigned to basic residues of the loop (K22A, K23A and R29A) for which the enterotoxigenicity decreased to about 65, 22 and 51%, respectively. The consequences of combining substitutions were also studied. The double mutants K22A-K23A and K23A-R29A showed enterotoxigenicities of about 17 and 7%, respectively. These values are lower than those for the corresponding single mutants. Small changes in toxicity were noted with D30A, for which the enterotoxigenicity was about 51%. For mutants G27P and G34A, the enterotoxigenicity was reduced to 20 and 13%, respectively.

DISCUSSION

In this study, we examined the role of specific amino acids of STb in recognition and binding to SFT and in the enterotoxigenicity determined in the rat loop assay. As the positively charged amino acids were previously

shown to be important for toxicity, they were selected and tested in this study for binding to SFT. The loop region (C21–C36) and hydrophobic α -helix were of special interest because they comprise respectively numerous charged and hydrophobic amino acids. As Sukumar *et al.* (1995) have determined the NMR solution structure for STb, it was possible to make a critical choice about amino acids pointing into the solvent that could play a role in SFT binding and toxicity (Sukumar *et al.*, 1995).

According to the highly basic pI (9.6) of STb and because of the anionic sulfate residue carried by SFT receptor, it is conceivable that some positively charged amino acid residues of STb could participate in bringing together and in binding to SFT. Previously, Fujii *et al.* (1994) demonstrated that the lysine residues K22 and K23 were important in the expression of the enterotoxigenic activity of STb. Dreyfus *et al.* (1992) described the role of arginine R29 in toxicity. Therefore, these basic residues were tested for their affinity toward SFT using alanine replacement mutagenesis. The binding assay was done in a buffer containing 150 mM NaCl to more accurately mimic the physiological state. The dissociating salt should also help to neutralize the surface charges found on STb toxin. Because a significant decrease was observed in binding activity with mutants K22A, K23A and R29A, the individual amino acid residues K22, K23 and R29 evidently have an important and equivalent implication in the attachment to SFT. Yet, K22 and K23 seem to have an important role in binding as the double mutant K22A-K23A exhibited significantly less binding

capacity to SFT than the double mutant K23A-R29A. Moreover, an additive effect in binding reduction was noted when K22 and K23 were co-mutated. The results obtained for the various mutants tested indicate that binding to SFT represents the initial event leading to the *in vivo* enterotoxicity observed. In fact, the reduction in toxicity was always either equal to or greater than the decrease in binding to SFT. This was expected, as the binding of a toxin to its receptor is the trigger step in pathogenesis leading to specific toxic consequences. Significantly, the lysine residue K23 seems to have an additional role in enterotoxicity as inferred by the dissociation observed for mutants K23A, K22A-K23A and K23A-R29A between the enterotoxic and binding activities. Discrepancies were noted between our results and those of Fujii *et al.* (1994), notably about the relevance of K22 for enterotoxicity. Their K22L mutant was shown to be less enterotoxic (10%) than the K22A mutant (70%) presented in this study. This divergence may be explained by the shorter side chain of the alanine of our K22A mutant compared to the longer leucine of Fujii's K22L mutant.

At position 30 inside the loop (C21–C36), substitution of the aspartate for either alanine or valine reduced the enterotoxicity while the attachment to SFT seemed to be favoured. The suppression of the negative charge present on the aspartate side chain may eliminate a repulsive force that could occur in the vicinity of the SFT-binding site. Indeed, SFT bears an anionic sulfate group. Moreover, the substantial difference observed in attachment between mutants D30A and D30V could result from the preferential presentation of SFT due to the larger size of valine versus alanine or to the more hydrophobic property of valine compared to alanine. In contrast, this mutation appears to alter a subsequent step in enterotoxicity. The mutant D30V reported by Dreyfus *et al.* (1992) showed a similar enterotoxicity level (39%) to the D30A mutant examined in this study (49%).

Because of the flexibility conferred by four glycines in the loop (C21–C36), we hypothesized that they would play a role in binding. Toxins such as α -haemolysin of *S. aureus* are known to display this characteristic, which facilitates membrane insertion. To test this hypothesis, glycines G24, G27, G31 and G34 were selected for mutagenesis. The results showed a decrease in both attachment and enterotoxicity (except for mutant G34A, which showed a binding of 118%). This reduction may be due either to the elimination of the flexibility or to the low space occupancy of the glycine residue. As observed for K23, G31 and G34 seem to play an additional role in enterotoxicity other than simply the attachment. This conclusion is inferred by the dissociation between the enterotoxic and the binding activities observed for mutant G31A (1% and 41%, respectively) and mutant G34A (13% and 118%, respectively).

Among other SFT-binding proteins, the FasG subunit of the 987P fimbriae was shown to bind to its SFT receptor (Choi & Schifferli, 1999). Specifically, residues R116,

K117 and K118 in alanine-scanning site-specific mutagenesis experiments have been shown to play a role in attachment to SFT. Thus, for STb and FasG, closely gathered basic residues appeared to interact with SFT, involving a salt-bridge interaction with the sulfate group of SFT (Choi & Schifferli, 1999). Similar observations were made with properdin, another SFT-binding protein (Holt *et al.*, 1990). In contrast, the flavonol 3-sulfo-transferase (ST) displays some similarities with the STb toxin. ST is an enzyme using 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as cosubstrate. Structurally, PAPS is closely related to SFT, both molecules having an acidic sulfate group linked to a sugar ring. Marsolais & Varin (1995) did site-specific mutagenesis on ST throughout an amino acid stretch known to bind the cosubstrate PAPS, ²⁷⁶RKGXXGDWK(N/T)XFT²⁸⁸. They demonstrated that residues R276, K277, G281 and K284 were involved in cosubstrate binding by using mutants. Those residues could correspond to K22, K23, G27 and R29, found within STb loop ²²KKGFLGVRDGTAGA³⁵. Marsolais & Varin (1995) also established that conservative replacements such as K284R have little effect on PAPS-binding activity.

To test the hypothesis concerning the implication of the hydrophobic α -helix in enterotoxicity according to the STb NMR structure, three major hydrophobic residues facing the solvent (F37, I41 and M42) were mutated (Sukumar *et al.*, 1995). These mutants demonstrated weak enterotoxicity in the rat loop assay, providing compelling evidence for the role of selected non-polar residues in pathogenesis. Chao & Dreyfus (1998) have shown that STb toxin promotes leakage of lipid vesicles, indicating local disruption or membrane insertion. Considering that amphipathic and hydrophobic helical structures are required for membrane insertion of the toxin, we postulated that F37, I41 and M42, belonging to the C-terminal hydrophobic α -helix, could be involved in the internalization pathway inferred by Chao & Dreyfus (1997).

Mutants F37K, I41S and M42S showed the same reduction in binding to SFT compared to the reduction in enterotoxicity. However, these results were difficult to reconcile with other results obtained in our laboratory. In fact, Rousset *et al.* (1998) have shown that the epitope recognized by STb is specifically a galactose residue linked to a sulfated group at position 3. This structure displays no hydrophobic region enabling an interaction with STb. In fact, galactose is comprised of alcohol groups and one hemiacetal group conferring a polar environment. From these observations, theoretically no direct hydrophobic interaction could occur between STb and SFT. To explain the involvement of hydrophobic amino acids in binding to SFT, a model was developed implying an oligomerization process between STb molecules. As demonstrated recently, STb enterotoxin forms hexamers and heptamers through hydrophobic bonds between C-terminal hydrophobic α -helix residues F37, I41 and M42 (Labrie *et al.*, 2001). Full STb structure integrity is necessary for the oligomerization process to take place. We suggest that there

is basic binding activity between STb and SFT with a STb–STb inter-binding process, in part via the hydrophobic α -helix. In this case, the propensity of hydrophobic groups to associate due to their repulsion from the aqueous solvent compels STb molecules to interact. This could explain the decrease in attachment observed with polar mutants located in the hydrophobic α -helix. Oligomerization is observed for the α -toxin of *S. aureus* following a preliminary binding either with a high-affinity protein receptor, or, more commonly, with ubiquitous low-affinity acceptors (phosphatidylcholine and cholesterol). As for STb, the *S. aureus* α -toxin contains a glycine-rich flexible motif (Menestrina & Ferreras, 1997).

The most prominent result of this study is the establishment of F37, I41 and M42 as important residues for binding and enterotoxicity activities. In addition, we have demonstrated the importance of charged amino acid residues present in the loop (K22, K23, R29 and D30) in the attachment to SFT and confirmed their role in enterotoxicity. Mutations of these residues strongly inhibited both binding and toxic activities. Moreover, these latter hydrophobic residues appear to be more important in both attachment to SFT and enterotoxicity than the previously identified charged amino acids situated in the loop. Taken together, the results indicate that hydrophobic amino acids are directly implicated in the oligomerization process that precedes the binding to SFT. Binding to SFT is due to interaction of SFT with the positively charged lysine and arginine residues of STb. Our study has also clearly established that the binding to SFT is a prerequisite step preceding fluid secretion, as a reduction in binding was concomitantly associated with an equivalent or greater decrease in toxicity.

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