Effect of Single Peptide Bond Scission by Trypsin on the Structure and Activity of Staphylococcal Enterotoxin B

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SUMMARY
The in vitro exposure of staphylococcal enterotoxin B (enterotoxin-T) to trypsin resulted in the formation within 30 min of a product (enterotoxin - T) unchanged in molecular weight, but with threonine as a second NH$_2$ terminus. Upon reduction of the -SS- bridge in enterotoxin-T, two fragments were separated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The peptide bond between Lys-97 and Thr-98 was unequivocally identified by automated sequence determination as the site of cleavage by trypsin. This Lys-Thr bond is positioned in the 21-residue disulfide loop of enterotoxin B, and enterotoxin-T is thus a "nicked" molecule (COLLIER, R. J., AND KANDL, J. (1971) J. Biol. Chem. 246, 1496-1503). Native enterotoxin B obtained directly from bacterial cell culture was also nicked to a small extent, but not at the trypsin-susceptible bond.

In the absence of denaturant the two reduced fragments did not separate and were thus held together by strong non-covalent forces. Enterotoxin B and -T demonstrated very similar amino acid composition, sedimentation in the ultracentrifuge, gel filtration on Sephadex G-50, and reducibility of the disulfide bond by dithiothreitol. They were also indistinguishable in the quantitative precipitin reaction, gave a reaction of complete identity in Ouchterlony immunodiffusion, and showed equivalent emetic activity in rhesus monkeys. Hence, no significant physicochemical or biological difference could be found between enterotoxin B and -T. Although there is an analogy between the structures and reactivity to trypsin of diphtheria toxin and enterotoxin B, it is unlikely that the staphylococcal enterotoxins undergo a comparable physiologival activation since the antigenic variant staphylo-

coccal enterotoxin A was found to be completely resistant to trypsin.

The enterotoxins elaborated by Staphylococcus aureus are simple proteins which cause emesis and diarrhea in a limited number of mammalian species. Enterotoxin B has been isolated in very pure form (1) and is composed of a single polypeptide chain with one disulfide bridge and no free sulfhydryl groups (2). The amino acid sequence of the molecule has been determined and the molecular weight computed from it is 28,500 (3). Several studies have been carried out attempting to relate structure to biological activity through the use of specific modification reagents (4-7). However, no class or specific member of a class of residues has yet been found essential for either serological or emetic activity. Those effects that have been observed were attributable to conformational alterations. Thus, we have investigated partial enzymatic breakdown as a technique for separating and identifying the antigenic and toxic sites of enterotoxin B. The activity of enterotoxin B has been reported to be destroyed by pepsin, ficin, and crude protease, but resistant to chymotrypsin, rennin, papain, and trypsin (1). We have observed, however, that native enterotoxin does undergo a limited digestion by trypsin and the preparation and properties of the product are described in this report. The primary site of cleavage is in the region of the disulfide bridge and the product obtained retains full biological activity.

EXPERIMENTAL PROCEDURE

Materials—Staphylococcal enterotoxin B was prepared by the method of Schantz et al. (1) except that the fermentation broth was desalted by passage through a Bio-Gel demineralization cartridge before dilution and adsorption onto CG-50; elution from this resin was carried out at pH 6.4 instead of pH 6.8. Enterotoxin A was prepared by the method of Schantz et al. (8). Enterotoxin C was prepared by the same procedure as enterotoxin B. Rabbit and goat antisera$^1$ were prepared as previously described (9, 10). L-(1-Tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin (Worthington) was used in all experiments. Soybean trypsin inhibitor was a three times crystallized product (Worthington). B-Mercaptoethanol (Eastman) was redistilled before use. Marker proteins were all crystalline and highly purified.

Methods—Enzyme digestions were carried out at 30°C in either 0.05 M Tris hydrochloride at pH 9.0 or on a pH-stat in 0.05 M KCl (Radiometer TTT1a) under water-saturated nitrogen and contained 0.01 M Ca$^{2+}$. The protein concentration was usually about 5 mg per ml. Reactions were stopped by the addition of a 1.5- to 2.0-fold excess of soybean trypsin inhibitor or by passage through a column of the inhibitor coupled to Sepharose 4B (11).

$^1$ In conducting the research described in this report, the investigators adhered to the Guide for Laboratory Animal Facilities and Care, as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council. The facilities are fully accredited by the American Association of Accreditation of Laboratory Animal Care.
Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out by the method of Weber and Osborn (12) on a Hoefer Scientific instrument. Samples were incubated at 50° for 2 hours in 0.01 M phosphate buffer at pH 7.0 containing 1% sodium dodecyl sulfate and 1% β-ME, and after being layered onto the gels, were made at least 4 M in urea by the addition of 8 M urea. The gels were stained for 2 hours with Coomassie blue and destained by diffusion in the solvents described by Greaser and Gergely (13). Gels were measured at 570 nm on a Gilford 240 spectrophotometer equipped with a linear transport device. Migration distances were measured from the centroid of the peaks on these gels and molecular weights were calculated by the method of Dunker and Ruedenberg (14) which relates migration to an internal protein standard rather than to a marker dye. The scans were also used to follow the kinetics of the conversion of enterotoxin B by trypsin. The concentration of residual enterotoxin was determined from the area of the peak measured by planimetry. It was established that enterotoxin B in gels followed Beer's law.

Amino acid analysis was done on a Technicon automatic amino acid analyzer. Terminal amino acids were identified as dinitrophenyl derivatives by the Levy modification of the method of Sanger (15). Automated Edman degradations were performed with a Beckman Sequencer, model 890 B in 1 M Quadrol buffer (16). The phenylthiohydantoins were identified by chemical ionization mass spectrometry (17) on a Finnigan mass spectrometer equipped with a PDP-8/e Digital computer and a Complot plotter. Isobutane was used as the carrier gas and the source was maintained at 200°. The samples were applied by a direct insertion probe and the probe was heated from 30° to 250° over a 90-s period (18).

Disulfide bond reduction of native and trypsinized enterotoxin B was performed essentially by Cleland’s method (19). Reduced viscosities were obtained by measurement at 24° in Cannon-Ubbelohde viscosimeters with a water outflow time of 250 s. Sedimentation constants were determined on a Spinco model E ultracentrifuge, equipped with a rotor temperature indicating and control unit, absorption optics, and an ultraviolet scanner. Tests for immunological and emetic activities were carried out as previously described (4).

RESULTS

Formation of Enterotoxin-T—When staphylococcal enterotoxin B was treated with trypsin a rapid initial reaction was observed in the pH-stat which slowed to a base rate after about 1.5 hour. Extrapolation of the base rate to zero time permits a computation of the amount of alkali consumed during the initial reaction. Assuming a pK₄ of the liberated amino groups of 7.6 (20), a value of 1.9 bonds hydrolyzed per molecule at pH 9.0 was calculated. At both pH 8.5 and 8.0, a value of 1.5 was obtained. These results indicate that the primary effect of trypsin is to cleave a single, specific peptide bond in the enterotoxin B molecule. The small additional titration is probably due to some secondary hydrolysis or extensive degradation of a small fraction of the preparation, or both.

It was initially anticipated that the enterotoxin B would be broken into two fragments following trypsin treatment. However, when the product, enterotoxin-T, was examined in the ultracentrifuge and by Sephadex G-50 gel filtration, only one component showing the same molecular size as untreated enterotoxin B was found. Disc electrophoresis in the presence of sodium dodecyl sulfate provides an explanation of this anomaly (Fig. 1). From a standard curve obtained by plotting the rate of migration against the logarithm of the molecular weight of a series of standard proteins (bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin, lysozyme, and chymotrypsin), enterotoxin B had a molecular weight of 28,000. In the absence of β-mercaptoethanol, enterotoxin B moved as a single line (gel 1). Enterotoxin-T also gave only a single line in the absence of reductant and it was located at the same position as the untreated enterotoxin (gel 3). In the presence of β-ME, however, enterotoxin-T (gel 4) had only a trace of material at the original migration site and two new, smaller peptides were seen. These fragments were estimated to have molecular weights of 16,400 and 13,000. Some variability in these values was obtained, but the sum of the molecular weights of the peptides was never more than 3% from the value of the molecular weight of enterotoxin B computed from its amino acid sequence. This deviation is well within the error of the method and indicates that the fragments are formed by a single peptide cleavage and together comprise the entire molecule.

Native enterotoxin B in the presence of β-ME showed a very low level of two smaller polypeptides (gel 2). An analogous...
phenomenon has been observed with diphtheria toxin (21, 22). Those molecules which demonstrate two moieties in the presence of reducant but only one in its absence are designated as nicked. Enterotoxin B then is naturally nicked to a small extent, presumably as a result of enzymatic action or chemical hydrolysis either during fermentation or subsequent purification. This has been observed in every enterotoxin B preparation examined. Enterotoxin-T is a totally nicked molecule. However, natural nicking is not identical with that brought about by trypsin, as shown in Fig. 2. The traces demonstrate that the peptides from the naturally nicked toxin are closer in size than those observed by tryptic cleavage.

If proteolysis by trypsin was allowed to proceed for longer periods (1 to 8 hours) a second product was formed with a molecular weight, estimated by gel electrophoresis, of 22,000. Since this material was detectable only in the absence of reducant it was formed subsequent to the formation of enterotoxin-T and was thus completely nicked. These materials were generated at vastly different rates. The formation of enterotoxin-T at several enzyme to substrate ratios is shown in Fig. 3. It is apparent that nearly quantitative conversion occurred in 1/2 hour at a substrate to enzyme ratio of 50:1. Only a small amount of the secondary product was observed at this enzyme level (side infra).

All subsequent studies were carried out on material consisting of at least 95% of the primary product and less than 5% of the secondary product.

Site of Cleavage—There are five possible sites of trypsin cleavage in the disulfide region of enterotoxin B, one Lys-Lys, one Lys-Arg, one Arg-Lys, and two Lys-Thr bonds (3). When enterotoxin-T was examined for NH2-terminal residues by the dinitrophenylation technique only DNP-threonine was found in significant yield in addition to the DNP-glutamic acid from residue 1. The yield of DNP-Thr was low, 0.33 mole per mole of enterotoxin. This was 48% of the yield of DNP-Glu which in these studies was 0.67 mole per mole. Although this is disturbing it should be noted that DNP-Thr has been found in low yield in at least one other instance, β-galactosidase (23), where 0.37 mole per mole were found. The Lys-Thr bonds in the disulfide loop (there are no other Lys-Thr or Arg-Thr peptides in enterotoxin B) are at positions 97-98 and 110-111. The location was confirmed by obtaining ratios of histidine to arginine in enterotoxin-B; - - - - , 100 µg of enterotoxin-B; - - - - - , 10 µg of enterotoxin-T.

Fig. 3 (right). The effect of trypsin concentration upon the rate of formation of enterotoxin-T. Digestions were carried out at pH 9.0 at 30°. Aliquots were added to excess soybean trypsin inhibitor and assayed after sodium dodecyl sulfate polycrylamide gel electrophoresis.

Fig. 2 (left). Comparison of sites of natural nicking of enterotoxin B with the product of tryptic action. Sodium dodecyl sulfate polycrylamide gels were stained with Coomassie blue and scanned spectrophotometrically. The molecular weights of the 1-97 and 98-239 fragments are 11,450 and 17,050, respectively. The molecular weights obtained on sodium dodecyl sulfate gel electrophoresis are more consistent with hydrolysis at position 110-111. However, it has been shown by several investigators that this technique for estimating molecular weights is least accurate in the low molecular weight region, with an abrupt departure from linearity at about 10,000 (14) where charge and shape play important roles in migration rate (24). The value obtained for the larger fragment, 98-239, is within 4% of the true figure, but the molecular weight of the smaller peptide 1-97 is off by 13%. The deviation is thus in the direction expected from the break in the standard curve.

Characterization of Enterotoxin-T—Analysis for free amino acids in the reaction mixture after removal of trypsin showed no amino acid in significant molar ratio to the enterotoxin. This precludes the formation of free amino acids or a small peptide along with the primary hydrolytic product at any position in the sequence. In addition, amino acid analysis of enterotoxin-T after hydrolysis in 6 N HCl was indistinguishable from that of enterotoxin B. If a second bond were also split in the disulfide loop in enterotoxin-T either free lysine, free arginine, arginyllysine, or a sizable peptide containing histidine or arginine, or both would be formed. The first three are ruled out by the direct amino acid analysis. The last possibility is conveniently checked by obtaining ratios of histidine to arginine in enterotoxin-T and between enterotoxin B and enterotoxin-T, since there are only 5 residues of each in the enterotoxin. No changes were found. Finally, when the product was examined by gel filtration on Sephadex G-50 only a single peak was obtained and no ninhydrin positive or ultraviolet light absorbing materials were found in the low molecular weight region of the chromatogram.

Enterotoxin-T was indistinguishable from enterotoxin B in velocity sedimentation experiments in the analytical ultracentrifuge. A very small increase in intrinsic viscosity was found in enterotoxin-T. Comparative data are shown in Fig. 4. Enterotoxin B is reduced quantitatively by a 700-nolar excess of β-mercaptoethanol in the absence of urea (4). It was not

2 Nearly equimolar quantities of asparagine and aspartic acid were found after the third Edman cycle. Partial deamidation has been shown to be responsible for the multiple components of enterotoxin B observed in isoelectric focusing (L. Spero, J. R. Warren, and J. F. Metzger, paper in preparation) and apparently Asn-100 is one of these labile residues.
The reduced viscosity of enterotoxin B and enterotoxin-T. Measurements were made at 24° in 0.2 M Tris-HCl at pH 8.0. • enterotoxin B; △—△, enterotoxin-T.

Gel filtration of reduced enterotoxin B and enterotoxin-T on Sephadex G-50. Proteins were reduced with β-ME and applied to a column (1.5 × 90 cm). The column was equilibrated and eluted with 0.2 M Tris-HCl at pH 8.0 containing 0.01 M β-ME. ———, enterotoxin B; ———, enterotoxin-T.

appreciably reduced, however, by 6 mM dithiothreitol at a 10-molar excess to enterotoxin B when denaturant was not present (0.2 ± 0.1 mole per mole). Similar behavior was observed for enterotoxin-T. These data indicate that no major conformational change around the SS-loop occurred in going from enterotoxin B to enterotoxin-T.

There is apparently a strong noncovalent interaction between the two fragments of enterotoxin-T. Thus, when it was reduced by 0.5 M β-ME and chromatographed on Sephadex G-50 with the solvent 0.01 M with respect to β-ME, no separation was seen. Fig. 5 compares the elution pattern for reduced enterotoxin B and enterotoxin-T. Both appear very shortly after the void volume. The second peak in the trace is due to the excess β-ME used in the reduction. It was found that the fragments were not dissociated even after exposure to 4 M urea for 4 days.

Biological Properties of Enterotoxin-T—Fig. 6 compares the quantitative precipitin curves of native enterotoxin with the trypsin-treated product. No difference is evident. Further more, a reaction of complete identity was seen in Ouchterlony immunodiffusion. Reduced enterotoxin-T also gave an essentially unaltered precipitin curve (Fig. 7). The curves show that reduction did not affect the formation of the immune precipitate of either enterotoxin B or enterotoxin-T and that there was no interference from the low level of β-ME employed in the precipitin tubes. This is not surprising in view of the evidence noted above of strong noncovalent interaction between the two fragments.

The emetic activity of enterotoxin-T was determined in rhesus monkeys and the results of the titration are shown in Table 1. The intravenous median effective dose (ED₅₀) for enterotoxin B has been found with a very large group of monkeys to be 0.1 μg per kg (1). The small group of animals exposed here to the native toxin gave 100% response at 0.3 μg per kg, demonstrating that they were representative animals and that the preparation used in this study was fully active. Obviously enterotoxin-T was equally efficacious.

Reaction of Trypsin with Other Enterotoxins—It was of interest to determine whether this sensitivity to trypsin cleavage is a property common to all the staphylococcal enterotoxins. Accordingly enterotoxin A and enterotoxin C₃ were treated with

Precipitation of rabbit antistaphylococcal enterotoxin B by enterotoxin B and enterotoxin-T. Samples were incubated for 4 hours at 37° and then overnight at 4°. • enterotoxin B; △—△, enterotoxin-T.

The effect of reduction upon the precipitation of rabbit antistaphylococcal enterotoxin B by enterotoxin B and enterotoxin-T. The proteins were reduced with 0.5 M β-ME and the reaction mixtures were 0.01 M in β-ME. •, enterotoxin B; O, reduced enterotoxin B; △—△, reduced enterotoxin-T.
the enzyme under the same conditions used successfully for enterotoxin B. Enterotoxin C1 behaved much like enterotoxin B. The untreated preparation was slightly nicked and it reacted rapidly with trypsin in the region of its disulfide bridge to form two peptides on reduction. These peptides were so close in molecular weight that they moved as a single zone on sodium dodecyl sulfate gel electrophoresis. The nicking in native enterotoxin C1 also produced only one zone. Enterotoxin A, however, was not nicked and appeared to be completely resistant to trypsin.

The secondary hydrolysis observed with enterotoxin B was also seen with enterotoxin C1. The product had an estimated molecular weight of 21,000 and was completely nicked. The enterotoxin C1 was much more labile in this respect than enterotoxin B. A comparison of the rates of formation of the first and second products for both is shown in Fig. 8. The peak areas of the unreacted enterotoxins and the products of secondary hydrolysis were determined from gels run without β-ME. In the
to the number of animals responding and the denominator refers to the total number of animals tested.

Table I

<table>
<thead>
<tr>
<th>Entero- toxin B</th>
<th>Diarrhea and emesis</th>
<th>Latent period</th>
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<td></td>
<td>0.1 µg/kg</td>
<td>0.3 µg/kg</td>
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<td></td>
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<td>110</td>
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*Where response is given as a fraction, the numerator refers to the number of animals responding and the denominator refers to the total number of animals tested.

contrary to earlier reports of the complete resistance of native staphylococcal enterotoxin B to tryptic hydrolysis, it was found that enterotoxin B undergoes a rapid limited digestion. Initial cleavage is restricted to a single bond in the disulfide region of the molecule so that the molecular weight is unchanged. The peptide bond split is between Lys-97 and Thr-98. The product, enterotoxin-T, is unaltered in its biological or serological activity and is very similar in physical properties to enterotoxin B. Two measures of conformational change were employed, viscosity and availability of the disulfide bridge for reduction (25). Only a small increase was observed in the former and none at all in the latter. Aside from the additional NH2-terminal residue no chemical changes were found.

The two peptide fragments of enterotoxin-T found upon reduction of the disulfide bridge remain held together as a single molecular entity by noncovalent interactions. Precedence for this is elegantly provided in the research of Richards (26) on pancreatic ribonuclease and Anfinsen (27) on staphylococcal nuclease. In both instances the combined derivatives were enzymatically active, while the peptide fragments alone were inactive. Conditions have not yet been established for the separation of the fragments of enterotoxin-T in a native state.

A question of obvious physiological importance is whether the tryptic cleavage of enterotoxin B represents a biological activation. Activation by limited proteolysis is extremely widespread in nature. Examples include blood coagulation (28), milk clotting (29), formation of angiotensin (30), procollagen conversion (31), zymogen activation (32), and insulin activation (33). The diversity of function of the active proteins is matched by the diversity in the roles played by the precursors. In many instances there is an advantage to the synthesizing cell in having an enzyme or hormone inactive during its intracellular residence; in others the precursor serves as a physiological reservoir until needed at a specific site or to counteract an injury. There does not appear to be an obvious advantage to bacteria to synthesize an inactive toxin precursor. Yet several of the antigenic variants of botulinum toxin are elaborated as inactive protoxins (34) or as toxins with a greatly reduced specific activity (35) and the e and f toxins of Clostridium perfringens are both produced as nontoxic precursors (36, 37). Unaltered diphteria toxin has no biological activity and even the totally nicked material is inactive against the bacterial protein synthesizing system. But the effect of specific cleavage in diphteria toxin is of enormous significance for the expression of its biological activity. One of the fragments possesses the enzymic activity, inactivation of elongation factor, EF-2, that is considered to be the cause of toxicity. Both fragments, however, are required for toxicity toward living cells. There is a striking structural analogy between enterotoxin B and diphteria toxin, both possessing extremely sensitive peptide bonds within a disulfide loop. The bonds are so labile that in both proteins the isolated native ma-
materials are partially nicked. Indeed, we have even observed slow nicking of enterotoxin B at refrigerator temperatures in the absence of added enzyme.

Unfortunately a direct test of this hypothesis for enterotoxin B is impossible at present because neither its site or mechanism of action is known. We have approached the problem by comparing three highly purified enterotoxins, A, B, and C, with respect to their reactivity to trypsin. All three antigenic types have the same physiological action and presumably therefore the same active site. If trypsin initiated biological activity, it would be expected that the change would be common to all types. Enterotoxin C1 does behave in a similar manner to enterotoxin B, but enterotoxin A is completely refractory. Activation by a trypsinlike proteolysis of a peptide bond in the disulfide region of the enterotoxin is therefore precluded. Studies are continuing in our laboratory with a view to separating the peptides formed by trypsin action on enterotoxin B and a determination of their role in the antigenic and emetic activity of this protein.

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