Biological Activities of the Peptides of Staphylococcal Enterotoxin C Formed by Limited Tryptic Hydrolysis*

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Staphylococcal enterotoxin C1 is converted to a doubly cleaved molecule by trypsin digestion with one of the scissions internal to the disulfide loop and one external to it (Spero, L., Griffin, B. Y., Middlebrook, J. L., and Metzger, J. F. (1976) J. Biol. Chem. 251, 5580–5588). The larger, disulfide-containing polypeptide ($M_r = 22,000$) exhibited excellent binding to antisera to the intact enterotoxin. The residual NH$_2$-terminal fragment ($M_r = 6,500$) also bound to this antibody, but only weakly. Only the COOH-terminal carboxamidomethylated moiety of the $M_r = 22,000$ polypeptide ($M_r = 19,000$) combined with anti-enterotoxin C$_1$. Both the $M_r = 22,000$ and $M_r = 6,500$ polypeptides could partially inhibit the binding of enterotoxin C$_1$ to its antibody in a competitive system. It is suggested that enterotoxin C$_1$ possesses three major antigenic determinants, two on carboxamidomethyl $M_r = 19,000$ and one on the $M_r = 6,500$ fragment. A significant degree of refolding to a native-like conformation is indicated for the $M_r = 22,000$ and the carboxamidomethyl $M_r = 19,000$ materials by (a) the strong binding of these polypeptides to anti-enterotoxin C$_1$, (b) the strong binding of enterotoxin C$_1$ to antibody to the $M_r = 22,000$ polypeptide, and (c) their circular dichroic spectra in the far ultraviolet.

The $M_r = 6,500$ polypeptide exhibited mitogenic activity, but not emetic activity. Conversely, the $M_r = 22,000$ polypeptide was able to induce diarrhea in rhesus monkeys, but was not mitogenic, suggesting that the active sites for these activities are widely separated on the enterotoxin molecule.

Two of the enterotoxins elaborated by certain strains of *Staphylococcus aureus*, enterotoxin B and enterotoxin C$_1$, have been shown to be susceptible to limited specific enzymic digestion by trypsin (1, 2). The primary cleavage in both instances occurs at a site on the polypeptide chain interior to the disulfide loop. A secondary cleavage also occurs which for enterotoxin C$_1$ goes rapidly to completion. The doubly cleaved molecule, enterotoxin C$_1$-$T_2$, may be represented in schematic linear form, where the indicated molecular weights were obtained from amino acid analysis:

\[
\begin{array}{c}
\text{Glu} \quad \text{Lys} \\
\text{Axx} \quad \text{Lys} \quad \text{Val} \\
\text{Gly}
\end{array}
\]

Both enterotoxin B-$T$ and enterotoxin C$_1$-$T_2$ retain all the biological activities of the parent toxins: binding to antibody, mitogenicity, and the induction of emesis and diarrhea. Moreover, enterotoxin C$_1$-$T_2$ behaves as a single particle with essentially unaltered conformation.

The extraordinary lability to limited tryptic hydrolysis does not appear to play a role in enterotoxicity. Although the site of nicking is in the disulfide loop and thus bears a structural similarity to that occurring with several of the bacterial exotoxins (3–5), a parallel biological activation does not take place (1). We have suggested (2) that the kind of lability exhibited by these enterotoxins is associated with β turn structures at the protein surface. The labile Lys–Thr bond in enterotoxin B was found by the Chou and Fasman procedure (6) for the prediction of secondary structure to be part of such a region. It was noted too that a naturally nicked bond in concanavalin A was placed by x-ray crystallography between the 2nd and 3rd residues of an exposed β turn (7). A similar situation with human β$_2$-microglobulin has come to our attention (8). A Tyr–Ser bond at positions 10 and 11 is unusually susceptible to cleavage by chymotrypsin. Application of the Chou and Fasman method (6) indicates that this serine residue is the first member of a tetrapeptide with a high probability for forming a β turn. These specific cleavages can provide a means of obtaining large well-defined polypeptide fragments and we report here on the association of the polypeptides of enterotoxin C$_1$ with the various biological activities of the parent enterotoxin. It is demonstrated that both major tryptic polypeptides of enterotoxin C$_1$-$T_2$ possess antigenic determinants, but that mitogenic activity is restricted to one fragment and emetic activity is restricted to the other.

**EXPERIMENTAL PROCEDURES**

Materials—Staphylococcal enterotoxin C$_1$ and C$_1$-$T_2$ were prepared as previously described (2). The $M_r = 22,000$ and $M_r = 6,500$ polypeptides of enterotoxin C$_1$-$T_2$ were separated on a column of Sepharose 6B (Pharmacia) in 6 M guanidine hydrochloride (Schwarz/Mann, ultrapure grade) (2). For some testing of biological activity, it was necessary to purify these two polypeptides further. The $M_r = 6,500$ polypeptide was rechromatographed under the same conditions. The single resultant fraction was dialyzed free of guanidine and passed through an affinity column of rabbit antibody to enterotoxin C$_1$ bound to Sepharose 4B (9). This was to remove any residual trace of intact enterotoxin C$_1$-$T_2$ re-formed by complementation of contaminating $M_r = 22,000$ polypeptide with the $M_r = 6,500$ fragment. The $M_r = 22,000$ polypeptide, although appearing as a single peak in the gel filtration used for its isolation, was significantly contaminated.

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with enterotoxin C1-T7. Rechromatographing this fraction three times, each time selecting the latter one-half to two-thirds of the peak, served to reduce the contamination to levels acceptable for assay.

After reduction with β-mercaptoethanol and alkylation with iodoacetamide, the \( M_r = 22,000 \) polypeptide was separated into its constituent peptides, Cam1 \( M_r = 4,000 \) and Cam \( M_r = 18,000 \) by chromatography on Sepharose 6B in 6 M guanidine hydrochloride. These reactions were carried out as previously described (2) except that the medium was 6 M with respect to guanidine hydrochloride.

**Preparation of Antiserum—** Anti-enterotoxin C1 was prepared by intracutaneous injection of the enterotoxin without adjuvant in New Zealand white rabbits. A regimen based on that developed by Silverman et al. (10) was employed. Only those sera giving identical Ouchterlony precipitin lines against enterotoxin C1-T7 were pooled. Rabbit antiserum for the \( M_r = 22,000 \) polypeptide was produced by intramuscular inoculation of three 100-μg doses of polypeptides at weekly intervals in 10% rabbit serum albumin. Most of the serum used in this study was from a bleeding of a single rabbit made 1 week after a second course of immunization administered 3 months after the first series of injections.

**1. Labeling of Enterotoxin C1 and Tryptic Peptides—** All labeling was carried out by the gaseous diffusion method of Gruber and Wright (11) with \(^{125}\)I. Enterotoxin C1 was labeled in phosphate-buffered saline. The peptides were dissolved in 6 M guanidine hydrochloride during labeling and unbound radioisotope was removed by dialysis against 0 M guanidine hydrochloride.

**RESULTS**

**Solubilization of Peptides—** Obtaining the peptides in a stable, soluble state from the concentrated guanidine hydrochloride solutions in which they were isolated was a significant problem, especially for the \( M_r = 22,000 \) fragment. Dialysis against aqueous buffers over a wide pH range resulted in precipitation of most of the polypeptide and an unstable solution. It was soluble in both anionic (sodium dodecyl sulfate) and cationic (cetylpyridinium chloride) detergent solutions but serologic activity could not be demonstrated in the presence of either detergent. Successful solubilization was achieved by dilution into concentrated solutions of bovine serum albumin. The general procedure consisted of the dropwise addition of a 5- to 10-mg/ml solution of the polypeptide in 6 M guanidine hydrochloride (obtained by dry concentration with Aquacide (Calbiochem)) to a vigorously stirred 10% buffered solution of the albumin. Concentrations up to 1 mg/ml were readily obtained and the solutions were stable for several days. Except where noted, all biological measurements were carried out on peptides prepared in this manner.

**Serologic Properties of the Tryptic Peptides—** The binding capacities of the radioiodinated major peptides of enterotoxin C1-T7, the \( M_r = 6,500 \) fragment, and the \( M_r = 22,000 \) fragment, are compared in Fig. 1 with that for intact \(^{125}\)I-enterotoxin C1. The data have been normalized to equal amounts of labeled antigen; this is based on the assumption that over the range of antigen employed, the ratio of antibody to antigen at the endpoint is independent of antigen level. It is readily apparent that the \( M_r = 22,000 \) polypeptide bound very well to the antibody to the whole enterotoxin; on a weight basis, the affinity was nearly one-half that of enterotoxin C1, and essentially all of the label was precipitable. The binding of the \( M_r = 6,500 \) polypeptide was significant but considerably weaker.

Competitive inhibition of these two polypeptide fragments with the native enterotoxin is presented in Fig. 2. Both materials competed successfully with the native toxin for its homologous antibody. As anticipated, the \( M_r = 22,000 \) polypeptide was the more potent inhibitor, but a plateau of reaction was not reached with either fragment at its highest available concentration. If it is assumed that each fragment contained a unique determinant, some antibodies for intact

\[ M_r = 19,000 \text{ but only 0.4 mg/ml with the } M_r = 22,000 \text{ fragment. At least 32 repetitive scans were run using the data processor at a time constant of 4 s. A cell with a path length of 0.1 mm was used in the far ultraviolet for all the peptides except the } M_r = 22,000 \text{ material where a 0.5-mm path length was employed.} \]

1 The abbreviation used is: Cam, carboxamidomethyl.

2 In conducting the research described in this report, the investigators adhered to the “Guide for the Care and Use of Laboratory Animals,” as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

**FIG. 1.** Binding of \(^{125}\)I-labeled enterotoxin C1, and of two \(^{125}\)I-labeled polypeptides derived from it by trypsin digestion to rabbit antiserum to enterotoxin C1. The data have been normalized to equal amounts of labeled antigen, enterotoxin C1; \( \square \), enterotoxin C1; \( \bullet \), \( M_r = 22,000 \) polypeptide; \( \bigcirc \), \( M_r = 6,500 \) polypeptide.
toxin would be incapable of combining with the fragment under assay and total inhibition of the native enterotoxin-antiserum reaction could not be achieved.

Neither of these polypeptide preparations contained $M_r = 28,000$ material by polyacrylamide gel electrophoresis. However, as noted earlier, the detectable limit of the method is slightly better than 0.1%. This does not present a problem for the $M_r = 22,000$ polypeptide because significant inhibition was observed at concentrations where contamination levels with whole enterotoxin of 1% would be necessary to produce the observed effect. Moreover, if the inhibitory response were indeed due to contamination, the inhibition curve would have been identical with that seen with enterotoxin C1, but displaced to the right (cf. Ref. 13, Fig. 6). With the $M_r = 6,500$ polypeptide, we must rely on the methods employed in its isolation and purification; after the second chromatographic separation, $M_r = 28,000$ material was not detected on analytic gels and subsequent purification with the antibody affinity column was demonstrated to be capable of removing enterotoxin contaminants several orders of magnitude greater than would be present.

These difficulties are obviated in the antigen-binding capacity assay where trace contamination cannot contribute significantly to the percentage of labeled antigen bound to the antibody. Binding data with antiserum to enterotoxin C1 and to the $M_r = 22,000$ polypeptide are shown in Table I for intact enterotoxin and its four tryptic peptides. In these calculations, it was assumed that all the antigen bound at the 50% endpoint was in the form Ag:Ab (15), so that for the bivalent antibody, the molar ratio of antigen to antibody was 4. Endpoints were estimated from log-log plots of the volume of antibody against the percentage of antigen bound. Antibody levels and molar ratios were calculated assuming that all antigen bound at the endpoint was in the form of Ag:Ab.

Table I

| Antigen | Molar ratio of total antibody to labeled antigen at 50% endpoint |
|---------|---------------------------------------------------------------|
| Enteotoxin C1 | Anti-$M_r = 22,000$ | Anti-enterotoxin C1 |
| $M_r = 6,500$ | 0.57 | 0.25* |
| $M_r = 22,000$ | 0.25* | 0.4 |
| Cam $M_r = 4,000$ | 4.3* | NBE* |
| Cam $M_r = 19,000$ | 1.2 | 1.5 |

* Fixed by computational assumptions.
* NBE, no binding evident.

Endpoint estimated by linear extrapolation of log-log plot.

The excellent binding of the $M_r = 22,000$ polypeptide to anti-enterotoxin C1 is indicative of a high degree of refolding of this fragment to a native conformation. This contention is supported by the surprisingly strong binding of the whole enterotoxin to antiserum raised against the $M_r = 22,000$ polypeptide. Enterotoxin C1 is a very stable, compact protein and the antibody sites with which it combines reflect immunoglobulin biosynthesis induced by determinants intrinsic to the intact native enterotoxin, i.e. these regions must also be present in the immunogen, the $M_r = 22,000$ polypeptide. It is well established that antibodies elicited by immunization with denatured protein either fail to react, or do not react extensively, with the native protein (16). Conversely, it is also clear that complete refolding does not exist in the $M_r = 22,000$ polypeptide. This was demonstrated by 1) the binding of Cam $M_r = 4,000$ for the antibody to the $M_r = 22,000$ polypeptide despite its complete failure to react with anti-enterotoxin C1 and 2) by the better binding of Cam $M_r = 19,000$ to the anti-$M_r = 22,000$ polypeptide than to anti-enterotoxin C1.

Emetic Activity of the Tryptic Peptides of Enterotoxin C1—
All the animals used for test of emetic activity of the tryptic polypeptides had no antibody titer to enterotoxin B or C1 by hemagglutination assay. No animals died and all appeared to be completely normal within 24 h after inoculation. The $M_r = 6,500$ polypeptide produced no emesis or diarrhea in monkeys injected intravenously with doses up to 10 $\mu$g/kg, the equivalent to 300 median effective (ED$_{50}$) doses of the intact enterotoxin. The results of assay of the $M_r = 22,000$ polypeptide in rhesus monkeys are shown in Table II. This prep-
aration of the polypeptide contained 0.25% contamination with $M_r = 28,000$ material which represents less than one-fourth of an ED$_{50}$ of enterotoxin at the higher dose of the $M_r = 22,000$ polypeptide at which diarrhea occurred. A positive response to this level of enterotoxin C$_1$ has not been observed. A positive result at the same level was obtained with another preparation of the $M_r = 22,000$ polypeptide in cynomolgus monkeys. It must be noted that no emesis was seen with this large polypeptide and the diarrhea was always less severe than with intact enterotoxin. We concluded that the active site for emesis and diarrhea is located within that portion of the molecule isolated on the $M_r = 22,000$ polypeptide.

The greatly reduced activity may be due to any of several factors operating singly or in combination, e.g. faster metabolic turnover, impaired conformation, or weaker binding to the putative receptor.

Mitogenic Activity of the Tryptic Peptides of Enterotoxin C$_1$—The $M_r = 6,500$ polypeptide demonstrated a low level of mitogenic activity. A typical response is shown in Fig. 3. In these experiments, the $M_r = 6,500$ polypeptide was prepared by removal of the guanidine in which it was isolated by dialysis against phosphate-buffered saline. No mitogenic activity was found for the $M_r = 22,000$ polypeptide under any of several solubilized conditions.

Circular Dichroism of the Peptides—In Fig. 4 are presented the circular dichroic spectra of the four tryptic peptides in the far ultraviolet. The spectrum of the native enterotoxin (2) in that region is included for comparison. The curves for the $M_r = 6,500$ polypeptide and for $M_r = 4,000$ are typical of random coil conformation. Both $M_r = 19,000$ and $M_r = 22,000$ fragments, however, show significant similarity to that of the intact enterotoxin. The spectrum of enterotoxin C$_1$ is very much like that of enterotoxin B which has been reported by analysis of its circular dichroism and by the Chou and Fasman procedure (6) for the prediction of secondary structure to contain about 30% $\beta$-pleated sheet and about 10% $\alpha$ helix (17). A striking feature of this structure is extensive grouping of anti-parallel $\beta$-pleated sheet around the disulfide loop. Much of this would be retained in both of these large polypeptides.

**DISCUSSION**

The assays for mitogenic and emetic activity of the two major polypeptides of enterotoxin C$_1$-T$_2$ indicate that these activities are associated with widely separated regions of the polypeptide chain. Mitogenesis was induced by the NH$_2$-terminal fragment, and diarrhea by the remaining portion of the molecule. We earlier suggested (14) that a residual mitogenic activity of the closely related enterotoxin B$_1$ detoxified by treatment with formaldehyde, implied that the mitogenic and emetic sites of this enterotoxin were not identical. The present studies point to a similar conclusion and provide a general localization of the sites responsible for these two activities on enterotoxin C$_1$.

Assay of antigen binding capacity with anti-enterotoxin C$_1$ demonstrated the presence of determinants (a) on the NH$_2$-terminal $M_r = 6,500$ polypeptide, (b) on the $M_r = 22,000$ polypeptide, and (c) on $M_r = 19,000$ (the COOH-terminal moiety of the $M_r = 22,000$ polypeptide), but not on $M_r = 4,000$ (the NH$_2$-terminal moiety of the $M_r = 22,000$ polypeptide). In competitive inhibition assays, a greater than 60% inhibition was achieved by the $M_r = 22,000$ polypeptide and 21% inhibition by the $M_r = 6,500$ polypeptide, suggesting that the larger polypeptide can combine with two-thirds of the antibody population and the smaller polypeptide with one-third of the antibody population. Assuming that the native molecule has three major antigenic determinants (quantitative precipitin analysis gives an effective antigen valence of 3 to 4), it is further inferred that the $M_r = 22,000$ polypeptide

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3 Some evidence of structural similarity of enterotoxins B and C$_1$ are: (a) a parallel susceptibility to the action of trypsin (1, 2); (b) serologic cross-reactions of the intact enterotoxins (13) and of structurally analogous tryptic polypeptides (L. Spero and B. A. Morlock, manuscript in preparation); (c) nearly identical CD spectra (J. L. Middlebrook, L. Spero, and P. Argos, manuscript in preparation); and (d) a high degree of homology in the amino acid sequence between the $M_r = 6,500$ polypeptide and $M_r = 4,000$ and the equivalent segments of enterotoxin B (J. S. Cades and L. Spero, unpublished observations).
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possesses two determinants (restricted to Cam \(M_1 = 19,000\)) and the \(M_2 = 6,500\) polypeptide one determinant.

The very good binding of the \(M_1 = 22,000\) polypeptide and the much weaker binding of the \(M_1 = 6,500\) polypeptide with anti-enterotoxin C1, is conveniently interpreted in terms of the concept of Sachs et al. (18) that the efficiency of binding of a combining fragment is a function of the equilibrium between native and disordered forms. In this light, the smaller polypeptide is largely in random conformation in solution, while the \(M_1 = 22,000\) polypeptide has an equilibrium greatly in favor of the native structure. CD spectra in the far ultraviolet, which indicated a lack of organized structure for the \(M_1 = 6,500\) material but were suggestive of a secondary structure for the \(M_1 = 22,000\) polypeptide similar to that of the intact enterotoxin, are consistent with this view. It is also supported biologically by the observation that the native enterotoxin binds extremely well to antibody to the \(M_1 = 22,000\) fragment. It may be noted that a significant degree of native structure on the \(M_1 = 22,000\) polypeptide also implies that the primary nucleation site for folding is in this part of the amino acid sequence. Further, it is contrary to the idea that virtually an entire sequence is required for folding to a native conformation from a disordered state (19). Wetlaufer and co-workers (20) have obtained a native-like structure from the peptide 13-105 of hen egg lysozyme.

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