Purification of Two *Clostridium perfringens* Enterotoxin-Like Proteins and Their Effects on Membrane Permeability in Primary Cultures of Adult Rat Hepatocytes

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We isolated two proteins, ET-1 and ET-2, from the sporangial extracts of *Clostridium perfringens* type A. Both proteins had some properties in common with the well-known *C. perfringens* enterotoxin. ET-1 and ET-2 behaved as single and distinct entities in anion exchange chromatography and disk gel electrophoresis. ET-2 was the more anionic protein since it eluted more slowly from the anion exchange column and migrated faster toward the anode in polyacrylamide disk gel electrophoresis (pH 8.5, native gels). Additionally, in this electrophoretic system ET-2 was not distinguishable from the enterotoxin. The amino acid compositions of ET-1 and ET-2 were similar but differed in a few amino acid residues. The values for both proteins were also similar to the published reports of others for the enterotoxin. Both ET-1 and ET-2 showed lines of identity in agar gel double immunodiffusion against anti-enterotoxin antiserum. Both ET-1 and ET-2 were toxic for rat hepatocytes in primary monolayer culture as determined by accelerated exodus of L-[¹⁴C]glucose from preloaded cells and by the rapid uptake of ⁴⁵Ca²⁺ after exposure to the proteins. In this regard, ET-1 and ET-2 appeared to be identical in mechanism of action to what has been regarded in the literature as "the" *C. perfringens* enterotoxin. Interestingly, ET-2 was 3 to 10 times more toxic on a weight basis than ET-1 was.

Certain strains of the bacterium *Clostridium perfringens* produce an enterotoxin which causes food poisoning in humans. Methods for purifying the enterotoxin have been reported (8-11, 18, 23). There is general agreement on the molecular weight (33,000 to 36,000), isoelectric point (pH 4.3), and amino acid composition of the enterotoxin. There have also been sporadic reports suggesting the possibility of multiple forms of the enterotoxin. For example (i) two forms not significantly different in molecular weight were detected by polyacrylamide disk gel electrophoresis (10); (ii) during chromatography a major and minor peak, both apparently containing biological activity, were detected, but these two peaks could not be distinguished by disk gel electrophoresis (23); (iii) two closely spaced bands seen occasionally in native gels were serologically active against anti-enterotoxin serum (2, 3, 20); and (iv) enterotoxin apparently chromatographically homogenous was resolved into two components by scanning isoelectric focusing and isotachopheresis (26).

Labbe and Rey (13) reported that adding raffinose to the culture medium results in increased

enterotoxin yields with some *C. perfringens* strains. The enterotoxin elaborated in raffinose-containing medium could not be distinguished antigenically from enterotoxin produced under previously described conditions in a medium without raffinose. Using this new procedure for enterotoxin production, we now report the preparation in milligram quantities of two proteins with some properties in common with what has been regarded in the literature as the *C. perfringens* enterotoxin. Both proteins are biologically active for rat hepatocytes in primary monolayer culture and appear to act through a mechanism identical with that previously reported for the *C. perfringens* enterotoxin (6, 7).

MATERIALS AND METHODS

Protein purification. Stock cultures of *C. perfringens* H-13 (NCTC 10240) were maintained at -20°C in cooked meat medium (Difco Laboratories, Detroit, Mich.). Spores were produced in DS sporulation medium (1) in which starch was replaced by 0.4% (wt/vol) D-raffinose pentahydrate (lot no. 23260, United States Biochemical Corp., Cleveland, Ohio) (13). Extraction of the enterotoxin from the spore-containing cells and all steps leading to the preparation of the enterotoxin

were done by a previously described method (23), except that chromatography with Cellex-T and hydroxylapatite were eliminated. These two steps were replaced by a single chromatographic step with a DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, N.J.) column (1.5 by 25 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 8.0. The sample was applied to the column, which was then washed with the equilibrating buffer until the absorbance of the fractions at 278 nm (A_{278}) was less than 0.01. A linear gradient of increasing molarity of phosphate was generated by mixing 250 ml of the equilibrating buffer with 250 ml of 0.04 M potassium phosphate buffer, pH 8.0. Enterotoxin-like protein in the eluted fractions was detected with an immunodiffusion technique (23). Antiserum was prepared against enterotoxin purified by the method of Stark and Duncan (23) without the modifications described here. Protein concentration was based on A_{278} and A_{260} (25). Polyacrylamide disk gel electrophoresis (nondenaturing) at pH 8.5 was performed as previously described (22). Densitometric tracings of the Coomassie blue-stained gels were made at 560 nm with a Gilford linear transport scanner (model 2410S).

Amino acid analysis. The purified proteins were extensively dialyzed against 0.1 M ammonium acetate and then lyophilized to remove water and ammonium acetate. Acid hydrolysis was performed in evacuated (25 mTorr) sealed tubes with 6 N HCl (double distilled) including 0.2% (vol/vol) phenol (88 to 90%) at 110°C for 24, 48, and 72 h. A Durrum 500 automatic amino acid analyzer was used. The error on this analyzer is $\pm 3\%$. Cysteine and cystine are reported as cystine. Tryptophan was not determined.

The number of amino acid residues was calculated as follows: Amino acids recovered from 24-, 48-, and 72-h hydrolyses were standardized to leucine (= 100), and with certain exceptions (see footnotes, Table 1) mean values were calculated. These numbers were then normalized to one of the least frequent residues, methionine (chosen over 1/2 cystine because methionine recovery was more consistent). The relative amounts of the amino acids, multiplied by their molecular weights (dehydrated), were added to derive the minimum residue weight. In the final step, the number of amino acid residues equaled [(molecular weight of the protein $\div$ minimum residue weight) $\times$ relative amino acid composition]. For these calculations, the molecular weights of ET-1 and ET-2 were assumed to be 34,000; this was also the weight assigned to the enterotoxin by others (8, 23).

Biological activity. Biological activity was assayed on primary monolayer cultures of adult rat hepatocytes (6, 7). Procedures for loading the cells with L-[^{14}C]glucose and determining the effect of enterotoxin on L-glucose exodus are described elsewhere (7). For determination of $^{45}\text{Ca}^{2+}$ uptake, dishes containing hepatocytes cultured for 48 h were used. The culture medium (7) was removed and the cells were washed once with Hank-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (7) at 37°C, and then Hank-HEPES buffer containing $^{45}\text{Ca}^{2+}$ (1 $\mu\text{Ci/ml}$; final Ca^{2+} concentration, 1.2 mM) was added. The plates were incubated in a shallow water bath at 37°C (7). After 5 min, ET-1 or ET-2 was added, and at various times thereafter plates were harvested and analyzed for intracellular radioactive label (7).

FIG. 1. Chromatography on DEAE-Sephacel column (1.5 by 25 cm) equilibrated in 0.01 M potassium phosphate buffer, pH 8.0, at room temperature. Flow rate, 40 to 60 ml/h; protein in fractions was detected spectrophotometrically at A_{278} . All buffers had 0.02% NaN_3 . (A) Load was 98 ml of crude enterotoxin preparation (A_{278} , 1.82; A_{260} , 1.13). Linear elution gradient, started at fraction no. 65, was generated by mixing 250 ml of the equilibrating buffer with 250 ml of 0.04 M potassium phosphate buffer, pH 8.0. Elution with 0.05 M potassium phosphate buffer containing 1.0 M NaCl (pH 7.0) was begun at fraction no. 157. Only the fractions under the two peaks marked ET-1 and ET-2 were serologically reactive to the anti-enterotoxin serum. Fraction size, 5.3 ml per tube. (B) The column was loaded with the material that was not held by the column in panel A, e.g., fractions no. 7 through 45 and corresponding fractions from similar columns. Load was 185 ml (A_{278} , 0.25). Step elution gradient was started at fraction no. 80 with 0.025 M potassium phosphate buffer, pH 8.0 (ET-1 and ET-2 could be eluted off the column shown in A with 0.025 M buffer). Fraction size, 5.4 ml per tube. (C) Rechromatography of ET-1. The column was loaded with fractions corresponding to ET-1 peak from two columns (see Fig. 2A and B); load was 83 ml (A_{278} , 0.16). Linear elution gradient, started at fraction no. 57, was generated with 240 ml of equilibrating buffer and 240 ml of 0.05 M potassium phosphate buffer, pH 8.0. Fraction size, 5.6 ml per tube. (D) Rechromatography of ET-2. The column was loaded with fractions corresponding to ET-2 peak from two columns (see Fig. 2A and B); load was 100 ml (A_{278} , 0.23). Linear elution gradient, as in C, was started at fraction no. 64. Fraction size, 5.3 ml per tube.

RESULTS

Purification and biochemical characterization of ET-1 and ET-2. The two protein fractions obtained with our purification methods were designated ET-1 (first peak containing apparent enterotoxin activity; Fig. 1) and ET-2 (second peak containing apparent enterotoxin activity; Fig. 1). Results of rechromatography (Fig. 1) showed that ET-1 and ET-2 were not artifacts of chromatography. Additionally, the data (Fig. 2) showed that other elution programs could also be used to separate ET-1 and ET-2. Hence, ET-1 and ET-2 could be isolated and separated by any of three related methods (Fig. 1 and 2).

FIG. 2. Chromatography on DEAE-Sephacel column (1.5 by 25 cm) equilibrated in 0.01 M potassium phosphate buffer, pH 8.0, at room temperature. Flow rate, 40 to 60 ml/h; protein in fractions was detected spectrophotometrically at A_{278} . All buffers contained 0.02% NaN_3 . Absorbance of the crude enterotoxin preparation at 278 and 260 nm was 6.17 and 4.09, respectively. Fraction size, 5.5 ml per tube. (A) Load, 62 ml. Elution with 0.025 M potassium phosphate buffer, pH 8.0, was started at fraction no. 63, and with 0.05 M potassium buffer containing 1.0 M NaCl, pH 7.0, at fraction no. 114. (B) Load, 33 ml. A linear elution gradient, started at fraction no. 59, was generated by mixing 80 ml of equilibrating buffer with 80 ml of 0.03 M potassium phosphate buffer, pH 8.0. From fractions no. 86 to 127 elution was done with 0.03 M potassium phosphate buffer, pH 8.0, and from fraction no. 127 to the end, elution was done with 0.05 M potassium phosphate buffer with 1.0 M NaCl, pH 7.0.

FIG. 3. Polyacrylamide disk gel electrophoresis of ET-1 and ET-2 immediately after their chromatographic isolation. Direction of migration is from top to bottom. Gels (7%) from left to right: ET-1 (A_{278} , 0.1; 40 μl); ET-2 (A_{278} , 0.17; 20 μl); mixture of ET-1 (80 μl) plus ET-2 (20 μl); and a mixture of ET-1 (40 μl) plus ET-2 (40 μl).

ET-1 and ET-2 migrated as separate and distinct entities in polyacrylamide gel electrophoresis (Fig. 3). We confirmed by gel scanning (not shown) that there were no bands in the gels other than those which were clearly discernible in the photographs (Fig. 3). The data (Fig. 1–3) supported the conclusion that ET-1 and ET-2 are homogeneous and distinguishable from one another, and that ET-2 is the more anionic protein. In other experiments (not shown), we found that ET-1 and ET-2 coeluted after chromatography through Sephadex G-100, indicating that they have similar molecular weights. We attempted analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to further investigate this issue, but the results were inconclusive due to apparent protein aggregation. Others have also reported similar anomalous aggregation of the purified enterotoxin in sodium dodecyl sulfate-polyacrylamide gels (3).

The amino acid compositions of ET-1 and ET-2 are shown in Table 1, along with similar data from the literature obtained with enterotoxin purified by previously described procedures. The two proteins which we isolated are similar except for a few amino acids. We do not yet know the biological significance of differences in amino acid composition, especially differences which appear to exceed 10% (proline, glycine, alanine, and histidine). The apparent lower histidine content of ET-2 may in part explain why it is more anionic than ET-1. With only a few

TABLE 1. Amino acid composition of *C. perfringens* proteins ET-1 and ET-2 and enterotoxin

Amino acid residue	No. of residues per molecule of protein ^a found by:			
	This study		Granum and Skjelkvale (8)	Hauschild et al. (11)
	ET-1	ET-2		
Aspartic acid	37	39	45	46
Threonine	22 ^b	23 ^b	21	19
Serine	38 ^b	39 ^b	34	31
Glutamic acid	27	28	29	28
Proline	9	8	9	9
Glycine	31	26	22	22
Alanine	37 ^c	32 ^c	16	16
Valine	15	14	17	18
Cysteine-Cystine	1 (0.5) ^d	1 (0.5) ^d	1 ^e	2 ^f
Methionine	1	1	2	3
Isoleucine	22	22	23	23
Leucine	25	26	29	29
Tyrosine	17	18	18	15
Phenylalanine	9	9	12	11
Lysine	16	16	17	16
Histidine	5	4	3	3
Arginine	7	7	7	7
Tryptophan	ND ^g	ND	3	3

^a Molecular weight of ET-1 and ET-2 was assumed to be 34,000; this value was used by Granum and Skjelkvale (8) for calculating amino acid composition. Hauschild et al. (11) estimated the amino acid composition based on the assumption of two cysteine/half-cystine residues per enterotoxin molecule; sum of amino acid residues yielded 33,200 as the molecular weight of the enterotoxin.

^b Extrapolated to zero time.

^c From 72-h hydrolysis.

^d Cysteine and cystine determined as cystine. The calculated value (0.5 residue) was rounded off to 1.

^e Determined as cysteic acid.

^f Determined as cysteic acid plus 1/2 cystine.

^g ND, Not determined.

exceptions, the amino acid contents found for ET-1 and ET-2 were very close to the values reported by others for the enterotoxin (Table 1).

We noted a shoulder on the trailing sides of the alanine peaks from both ET-1 and ET-2. The shoulder declined with hydrolysis time (the alanine values reported in Table 1 are from the 72-h hydrolysis, in which the shoulder on the alanine peak was minimal). This shoulder could possibly be due to glucosamine, which is known to co-elute with alanine in this system and is also acid labile (17).

An experiment (Fig. 4) was conducted to determine the extent of antigenic similarity between ET-1 and ET-2 when tested with antiserum previously prepared against enterotoxin purified by the method of Stark and Duncan (23) (and which neutralizes enterotoxin activity [6, 7]). ET-1 and ET-2 could not be distinguished by this technique. Hence, ET-1 and ET-2 appear to

contain antigenic sites shared in common with one another and with the enterotoxin. The lack of a spur is evidence against additional antigenic sites shared only by the enterotoxin and either ET-1 or ET-2.

Another experiment (Fig. 5) showed that ET-2 and the major component of enterotoxin purified by the method of Stark and Duncan (23) could not be distinguished electrophoretically. The data also showed that the enterotoxin preparation was not homogeneous and contained a minor component which was less anionic. In our previously published reports (6, 7) enterotoxin preparations were repurified until the minor (less anionic) component was no longer detectable. However, this may not have been done in some mechanism-of-action studies by others (see Fig. 1 in reference 16). It is possible that the less anionic component is actually ET-1, but not enough of this material was available for the necessary analysis to establish or disprove this conjecture.

Mechanism-of-action studies. We have previously reported on the mechanism of action of *C. perfringens* enterotoxin (6, 7). Our studies indicated that the enterotoxin rapidly attacks the membranes of sensitive cells and alters permeability. After exposure to enterotoxin, cations readily cross the plasma membranes of sensitive cells, as does L-glucose, which is not metabolized and for which there is no transport system (14, 24).

ET-2 effected a rapid and dose-dependent alteration in membrane permeability in adult rat

FIG. 4. Ouchterlony double diffusion test of ET-1 and ET-2 against antiserum prepared from enterotoxin purified by the method of Stark and Duncan (23). Plates were made of 1.5% agar in 0.05 M sodium phosphate buffer, pH 7.5, with 0.85% NaCl and merthiolate (1:1,000). Central well had the antiserum. Absorbance of ET-1 (well 1) and ET-2 (well 2) at 278 nm was 0.08 and 0.12, respectively. All wells were 4 mm in diameter and loaded with 20 μ l of sample.

FIG. 5. Comparison by polyacrylamide gel electrophoresis of ET-2 with the *C. perfringens* enterotoxin prepared by the method of Stark and Duncan (23). Gels (7%) from left to right: the *C. perfringens* enterotoxin (A_{278} , 0.36; 15 μ l); mixture of the enterotoxin (15 μ l) plus ET-2 (A_{278} , 0.27; 15 μ l); and ET-2 (15 μ l). The faint band at 28 mm of the two gels loaded with the enterotoxin represents an impurity of unknown nature; it could be removed by successive repurification with the final column step described in reference 3.

hepatocytes in primary monolayer culture (Fig. 6). We (7) have reported similar results for cultured hepatocytes treated with enterotoxin purified by the procedure of Stark and Duncan (23).

Figure 7 shows extensive dose-response data for ET-1 and ET-2, with $^{45}\text{Ca}^{2+}$ uptake and loss of L-[^{14}C]glucose (from preloaded cells) in primary cultures of adult rat hepatocytes used as the assay endpoints. The data indicated that ET-1 and ET-2 were biologically active, and that they both acted by altering membrane permeability. Moreover, on a weight basis ET-2 was about 3 to 10 times more active than ET-1 (Fig. 7); i.e., where ET-2 had an effect at a given concentration, 3 to 10 times as much ET-1 was required to produce the same effect.

DISCUSSION

C. perfringens enterotoxin is associated with the spore coat of the microorganism (4, 5) and is produced only in media which promote sporulation (12). Carbohydrates influence sporulation, and presumably for this reason raffinose increases enterotoxin production in strain H-3 by 14-fold (13). Previously reported purification procedures began with spores produced in

starch-containing DS sporulation medium (1), and the amounts of enterotoxin-like protein detected were low, probably too low to purify in quantity. In contrast, the procedures which we have employed result in relatively high yields of electrophoretically homogenous enterotoxin-like proteins. Therefore, it was possible for us to study in depth the physicochemical properties of these proteins as well as their mechanism of action.

Of the two enterotoxin-like proteins which we isolated and characterized, ET-2 was the more anionic, since it eluted more slowly from the anion exchange column and migrated faster towards the electrode in gel electrophoresis at pH 8.5 (Fig. 1-3). The amino acid compositions of ET-1, ET-2, and the enterotoxin were all similar (Table 1). Electrophoretically, ET-2 and the enterotoxin appeared to be more closely related (Fig. 5). ET-1 and ET-2 were serologically indistinguishable when tested against antiserum prepared against the enterotoxin (Fig. 4), and the data (Fig. 6 and 7) indicated that ET-1 and ET-2 were both biologically active and acted in similar ways on rat hepatocytes in primary monolayer culture. Like the enterotoxin (7), ET-1 and ET-2 both damaged the cell membrane, as indicated by the finding that the passive permeation of L-glucose was increased in cultures treated with ET-1 or ET-2 (Fig. 7). We also showed that $^{45}\text{Ca}^{2+}$ uptake was greatly increased in cultured hepatocytes treated with ET-1 and ET-2 (Fig. 7). This is the first report of enterotoxin-like protein effects on $^{45}\text{Ca}^{2+}$ uptake in sensitive cells and

FIG. 6. Effect of ET-2 on membrane permeability of adult rat hepatocytes in primary monolayer culture. (A) Passive exodus of L-glucose from preloaded hepatocytes. Hepatocytes treated with ET-2 at 3.3 (Δ) and 1.0 ($\circ$) $\mu\text{g}/\text{ml}$; control cells not treated with toxin ($\bullet$). (B) Uptake of $^{45}\text{Ca}^{2+}$. Hepatocytes treated with ET-2 at 1.0 (Δ) and 0.3 ($\circ$) $\mu\text{g}/\text{ml}$; control cells not treated with toxin ($\bullet$). Data points are means of three replicate cultures, with the standard error of the mean shown where it exceeds the size of the symbol.

FIG. 7. Effect of different concentrations of ET-1 (●) and ET-2 (○) on the passive exodus of L-glucose (determined 30 min after toxin addition) or the uptake of $^{45}\text{Ca}^{2+}$ (determined 10 min after toxin addition). Data points are means of three replicate cultures, with standard errors of the mean shown where they exceed the size of the symbol.

extends the previous report of Matsuda and Sugimoto (15) that morphological changes effected by *C. perfringens* enterotoxin are less severe in medium free of calcium. A rapid influx of calcium is apparently involved in cell death (19) effected by at least some toxins (21). Interestingly, on a weight basis ET-2 is 3 to 10 times more active than ET-1 (Fig. 7). Further investigation of the similarities and differences between ET-1 and ET-2 may prove fruitful in ultimately determining how *C. perfringens* enterotoxin and enterotoxin-like proteins interact with the membranes of sensitive cells to produce toxicity.

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