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Vibrio cholerae Hemagglutinin/Protease Nicks Cholera Enterotoxin

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Unnicked cholera enterotoxin was isolated from culture supernatants of *Vibrio cholerae* 569B by either rapid processing of flask-grown cultures or by growing and processing fermentor cultures in the presence of ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra acetic acid, an inhibitor of the previously described *V. cholerae* hemagglutinin/protease. When unnicked cholera enterotoxin was incubated with purified hemagglutinin/protease, the unnicked A subunit was converted to a molecular weight consistent with that of the A_1 subunit as demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and its specific activity for Y1 adrenal cells increased.

The biologically active A region of the *Vibrio cholerae* enterotoxin (choleragen or cholera toxin) is composed of two peptides, A_1 and A_2, derived from the A subunit by a single proteolytic cleavage (8). Under the usual conditions of isolation of cholera enterotoxin, the A subunit is in the nicked (activated) form, whereas the A subunit of the cholera-related heat-labile enterotoxin from *Escherichia coli* is largely in the unnicked form, which can then be activated by proteolytic processing (2).

We have previously reported (4) that overnight hydrolysis of unnicked *E. coli* heat-labile enterotoxin by the hemagglutinin (HA)/protease isolated from *V. cholerae* (6) yields a molecule consistent with the size change seen on activation of the heat-labile enterotoxin. We speculated that the HA/protease might be the endogenous enzyme of *V. cholerae*, which nicks and thus activates the cholera enterotoxin in a similar manner; however, to demonstrate this, we needed a cholera toxin with an unnicked A subunit.

Small amounts of unnicked cholera toxin, along with nicked cholera toxin, have been recovered previously from *V. cholerae* grown in the presence of phenylmethylsulfonyl fluoride (11) or in the presence of lima bean trypsin inhibitor (8). However, since we recently reported that the soluble HA/protease is a metalloenzyme (1) that can be inhibited by a variety of chelating agents and inhibitors of zinc metalloproteases, it seemed possible that unnicked cholera toxin could be isolated (in the absence of nicked toxin) by growing *V. cholerae* in the presence of one of these inhibitors.

In the present study, we show that it is possible to prepare large amounts of unnicked cholera toxin from fermentor-grown cultures of *V. cholerae* 569B when the cultures are grown and processed in the presence of ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Unnicked cholera toxin can also be prepared, in smaller amounts, in the absence of EGTA by growing cultures in flasks and then rapidly processing the material. This report also shows that the HA/protease nicks cholera enterotoxin A subunit and increases its biological activity.

MATERIALS AND METHODS

The purification of the HA/protease from *V. cholerae* CA401 has been described previously (6). Trypsin was purchased from Sigma Chemical Co. Cholera enterotoxin was partially purified from *V. cholerae* 569B Inaba through the stage of the TEAN (0.05 M Tris, 0.2 M NaCl, 1 mM EDTA, 3 mM NaN_3 [pH 7.5]) buffer elution of aluminum hydroxide-adsorbed material (5). Standard cholera toxin used as reference was fully purified (5). Cultures were grown overnight at 30°C in 200 ml of syncase medium in 1-liter Erlenmeyer flasks with reciprocal shaking or in 9.5-liter fermentors with vigorous aeration as previously described (5). Filter-sterilized EGTA (Sigma) was included in the fermentors at 2.5 mg/ml (6.6 mM) and at 0.4 mg/ml (1 mM) during processing. HA/protease activity was also inhibited by using Zincov [2-(*N*-hydroxy carboxamido)-4-methyl pentanoyl-L-Ala-Gly-NH_2] (Calbiochem-Behring). Approximate MICs of protease inhibitors for *V. cholerae* CA401 and 3083 were determined by serially diluting inhibitors in tryptic soy broth (Difco Laboratories) inoculated with 10^5 viable cells per ml. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (10), using 12% polyacrylamide gels. Molecular weight marker proteins (Bio-Rad Laboratories) were lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), and phosphorylase b (92,500).

Bioassays for cholera enterotoxin activity were done with Y1 adrenal cells in F-10 medium containing 10% fetal calf serum as described previously (13), using serial dilutions of toxin. The endpoint was the lowest concentration of toxin that caused readily observed rounding of the adrenal cells. Assays were performed in duplicate, and, in our experience, twofold differences are significant and reproducible. The concentration of enterotoxin was estimated by Mancini-type radial immunodiffusion (7), using equine anticholeragenoid (3). There was some error because the amount of choleragenoid relative to holotoxin is unknown.

RESULTS

To isolate unnicked cholera toxin, we initially evaluated the effect of the HA/protease inhibitor EGTA by using flask-grown cultures of *V. cholerae*. EGTA (MIC, 3 mg/ml) was chosen because it is a much better inhibitor of HA/protease than is EDTA (1) and because other strong inhibitors such as 8-hydroxyquinoline and *o*-phenanthroline are toxic to *V. cholerae* at low concentrations (MICs, 12.5 and 3.9 μg/ml, respectively).

Cultures grown in flasks in the presence of 2.5 mg of EGTA per ml reached viable cell counts of 3×10^9/ml, whereas those grown in the absence of EGTA reached 2 ×

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10^{10}/ml. Partially purified enterotoxin was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 12% polyacrylamide gels. A significant proportion of the A subunit was unnicked when flask-grown cultures with or without EGTA (or 150 μg of Zincov per ml [MIC, 3 mg/ml]; data not shown) were rapidly processed, i.e., within about 6 h (Fig. 1, lanes 8 and 9). When toxin was produced in fermentor-grown cultures that were then processed at room temperature during the next 2 days, the enterotoxin produced and processed in the absence of EGTA was almost completely nicked, whereas the enterotoxin isolated from the culture grown in the presence of 6.6 mM EGTA and processed in the presence of 1 mM EGTA was almost completely unnicked (Fig. 2). Thus, the chelating agent protected the cholera toxin from being nicked. However, when this unnicked, partially purified cholera toxin was left in TEAN buffer in the presence of 1 mM EGTA for 3 weeks at 4°C, the unnicked cholera toxin was largely converted to the nicked form.

To determine whether HA/protease could cut unnicked cholera toxin, the toxin, at 540 μg/ml, was incubated for 1.5 h at 37°C or overnight at 24°C with 16 μg of the purified HA/protease or with 1.8 μg of trypsin per ml. With either enzyme, the unnicked cholera toxin was converted to a molecular weight consistent with that of the nicked, activated form (Fig. 3). We compared the activity of the HA/protease-nicked toxin to the activity of the unnicked toxin by using the Y1 adrenal cell assay. Since overnight incubation of the Y1 cells with HA/protease alone led to cell rounding, Zincov was added to the protease-treated and control cholera toxin samples to neutralize the protease activity before the Y1 assay was run. Zincov alone had no effect on the Y1 cell assay. The effective dose of the partially purified unnicked cholera toxin was 98 pg, whereas the effective dose of the protease-treated toxin was 24 pg. The effective dose of the standard, purified (nicked) cholera toxin was 6 pg. The protease-treated toxin was not (apparently) as active as the

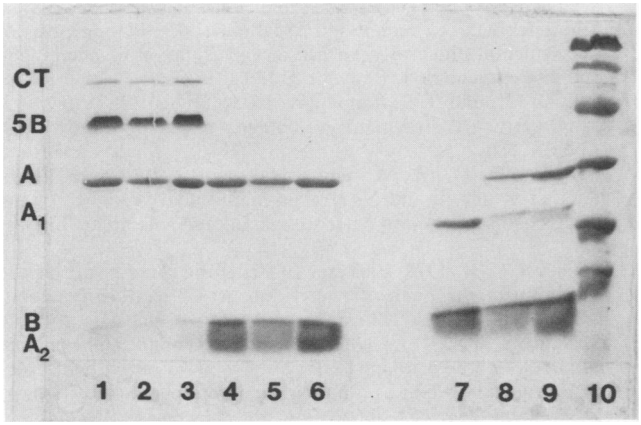

FIG. 1. *V. cholerae* cultures were grown overnight at 30°C in 200 ml of syncase medium in 1-liter Erlenmeyer flasks with reciprocal shaking in the presence or absence of 2.5 mg of EGTA per ml. *V. cholerae* enterotoxin was partially purified as described in the text, and the material was electrophoresed on 12% polyacrylamide gels containing sodium dodecyl sulfate. Samples in lanes 1 to 3 were unheated, and samples in lanes 4 to 6 were heated at 100°C for 5 min; in lanes 7 to 9, samples were reduced and heated. Lanes: 1, 4, and 7, standard purified cholera toxin; 2, 5, and 8, cholera toxin prepared from cultures grown in the presence of EGTA; 3, 6, and 9, cholera toxin prepared from cultures grown in the absence of EGTA; 10, molecular weight markers. Although toxin prepared from EGTA-supplemented cultures appeared to be less nicked than did the control (compare lanes 8 and 9), in both of these rapidly processed preparations, the A subunit was substantially unnicked. CT, Cholera holotoxin; 5B, pentameric B subunit (choleragenoid); A, A subunit; A_1, A_1 peptide; B, B subunit; and A_2, A_2 peptide (which frequently neither stains nor photographs well).

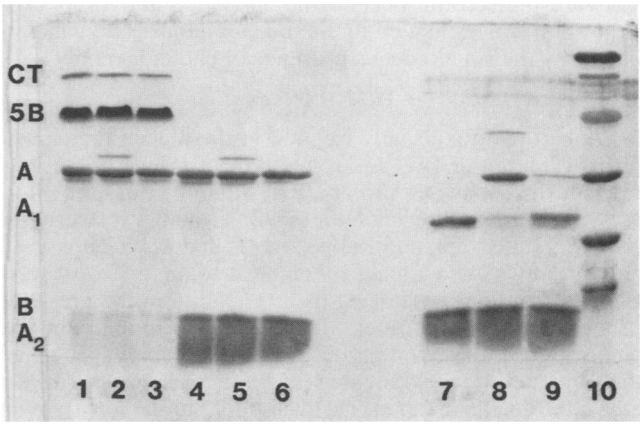

FIG. 2. *V. cholerae* cultures were grown overnight at 30°C in a 9.5-liter fermentor in syncase medium in the presence or absence of 2.5 mg of EGTA per ml. *V. cholerae* enterotoxin was partially purified as described in the text, and the material was electrophoresed on 12% polyacrylamide gels containing sodium dodecyl sulfate. Samples in lanes 1 to 3 were unheated, and samples in lanes 4 to 6 were heated at 100°C for 5 min; in lanes 7 to 9, samples were reduced and heated. Lanes: 1, 4, and 7, standard purified cholera toxin; 2, 5, and 8, cholera toxin prepared from cultures grown in the presence of EGTA; 3, 6, and 9, cholera toxin prepared from cultures grown in the absence of EGTA; 10, molecular weight markers. EGTA largely prevented nicking of the A subunit (compare lanes 8 and 9). The extra band above A in lanes 2, 5, and 8 (produced in fermentor-grown cultures in the presence of EGTA) has not been identified. Bands are labeled as for Fig. 1.

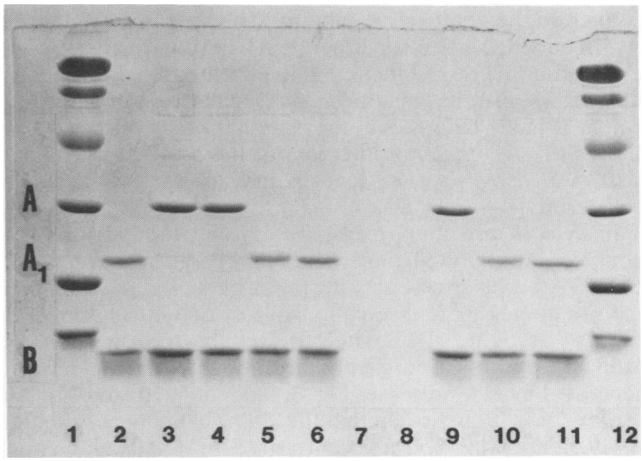

FIG. 3. Treatment of unnicked cholera toxin with proteases. All samples were reduced and heated at 100°C for 5 min. Lanes: 1 and 12, molecular weight markers; 2, standard purified cholera toxin (nicked); 3, unnicked cholera toxin kept at 4°C overnight; 4, unnicked cholera toxin kept overnight at 24°C; 5, unnicked cholera toxin plus 16 μg of HA/protease per ml kept overnight at 24°C; 6, unnicked cholera toxin plus 1.8 μg of trypsin per ml kept overnight at 24°C; 7, HA/protease as used in the incubations; 8, trypsin as used in the incubations; 9, unnicked cholera toxin incubated for 1.5 h at 37°C; 10, unnicked cholera toxin plus HA/protease incubated for 1.5 h at 37°C; 11, unnicked cholera toxin plus trypsin kept at 1.5 h at 37°C.

standard preparation because (as indicated above) its concentration was estimated by a crude immunoassay, which is biased by the simultaneous presence of choleragenoid (3, 7).

DISCUSSION

Cholera toxin is usually isolated in the nicked (activated) form. Addition of EGTA, an inhibitor of HA/protease, can prevent the nicking of cholera toxin subunit A in fermentor-grown cultures of *V. cholerae* 569B, although it decreases growth slightly. Gill and Rappaport (8) and Mekalanos et al. (11) isolated some unnicked cholera toxin by short-term growth and rapid processing of *V. cholerae* in the presence of serine protease inhibitors (lima bean trypsin inhibitor and phenylmethylsulfonyl fluoride, respectively) and speculated that the endogenous nicking enzyme was a trypsin-like enzyme. Melakanos et al. (11) were able to demonstrate an apparent conversion from A to A_1 with trypsin or elastase, although elastase further degrades some of the A_1 subunit. Gill and Rappaport (8) found that excess trypsin treatment destroys the biological activity of cholera toxin. Preparations of HA/protease (from *V. cholerae* CA401) do not appear to contain trypsin-like or other serine proteases; synthetic substrates for trypsin and other serine proteases (*p*-tosyl arginine methyl ester, *p*-tosyl lysine methyl ester, and *p*-nitrophenyl phosphate) are not hydrolyzed by HA/protease preparations (unpublished data), and trypsin inhibitors and other serine protease inhibitors do not affect the activity of the protease on ^{125}I-bovine serum albumin digestion (1).

Addition of HA/protease to unnicked cholera toxin yielded protein bands whose migrations in sodium dodecyl sulfate-polyacrylamide gel electrophoresis were identical to those of conventionally prepared (nicked) toxin. This protease-treated material had increased biological activity. Thus, HA/protease was capable of nicking and activating cholera toxin. Inhibition of this enzyme prevented nicking, which suggests that HA/protease is an endogenous *V. cholerae* "nickase" (if not the only endogenous nickase). Trypsin also converted the unnicked A subunit to a size similar to that of A_1. In our experiments, neither the HA/protease nor trypsin, even after overnight incubation, appeared to degrade A_1 further. Trypsin has also been used to activate unnicked *E. coli* heat-labile enterotoxin for quantitation by Y1 cell assay (2). During *V. cholerae* infection of the small bowel, other host proteases such as trypsin may contribute to toxin activation.

In light of previous results (8, 11) and the fact that the unnicked A subunit of cholera toxin prepared in the presence of EGTA broke down to A_1 during storage, it is possible that the site of nicking is especially labile or that other proteases can cut at or near this site. In fact the trypsin cleavage product appears to be slightly smaller than the A_1 subunit generated by HA/protease (Fig. 3, lanes 5, 6, 10, and 11). An earlier study (9) indicated that the amino terminus of subunit A_2 is methionine, and a recent report by Mekalanos et al. (12) indicates that the cleavage site of subunit A is, more precisely, between serine 194 and methionine 195. At two peptide bonds upstream (12) from the cleavage site is an Arg-Ser bond, a likely site of trypsin hydrolysis that would yield a slightly smaller A_1 fragment. It is also possible that other proteases may effect activation by nicking neighboring peptide bonds.

In summary, although gut enzymes may play a contributory role in toxin activation, the production of the soluble HA/protease, an endogenous nickase, provides *V. cholerae* with its own certain mechanism for toxin activation.

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