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The Arrangement of Subunits in Cholera Toxin†

D. Michael Gill

ABSTRACT: Cholera toxin consists of five similar B subunits of apparent molecular weight about 10 600 and one A subunit (29 000) consisting of two peptides (A_1 23 000–24 000 and A_2 about 5500) linked by a single disulfide bond. Each B subunit also contains one internal disulfide bond which is readily reduced but is protected from carboxymethylation unless the reduced subunits are heated in urea. Tyrosine residues in A_1 and in B subunits are readily iodinated, but the intact B assembly does not react with iodine. Upon reaction with the cross-linking reagent dimethyl suberimidate, B subunits may be covalently connected to each other, to A_1 and to A_2. A_1 and A_2 may also be cross-linked. The B subunits are probably arranged in a ring with A on the axis. A_2 is required for the re-assembly of toxin from its subunits and may serve to hold A_1 on the B ring. The maximum activity of cholera toxin in vitro is obtained only when the active peptide, A_1, is separated from the rest of the molecule. Such separation, and the insertion of A_1 into the cytosol, must follow the binding of the complete toxin, through component B, to the exterior of intact cells. This binding increases the effective concentration of the toxin in the vicinity of the plasma membrane. Possible ways in which A_1 then crosses the membrane are considered in the Discussion.

Recent evidence has changed our concept of the mode of action of cholera toxin. This protein can interact with intact vertebrate cells of many types and elevate the activity of the membrane enzyme adenylate cyclase (reviewed by Pierce et al., 1971; Finkelstein, 1973). It was formerly considered possible that the toxin might act stoichiometrically while bound to, or present in, the plasma membrane. However, for the past 2 years it has been possible to activate adenylate cyclase in disrupted cells and thereby analyze in some detail the events following addition of toxin. It is becoming increasingly clear that the toxin must act enzymically, that it catalyzes some intracellular reaction between one or more cytoplasmic compounds and that portion of the adenylate cyclase which protrudes into the cytosol.

The most rapid activation of adenylate cyclase in vitro requires the presence, apart from cell membranes, of NAD, ATP, and an unidentified soluble cellular protein (Gill, 1975a, 1976). Cholera toxin then changes the adenylate cyclase in such a way that the enzyme's activity rises considerably (at least 30-fold in the case of pigeon erythrocyte ghosts), its stability increases (Gill and King, 1975), its response to epinephrine is enhanced (Field, 1974), and the extent of its stimulation by fluoride ions is reduced (Sharp et al., 1973; Field, 1974). None of these changes are reversed by avid antibodies directed against the toxin.

Of the several peptides that compose cholera toxin, only peptide A_1 is required for the activation of adenylate cyclase in vitro (Gill and King, 1975). The adenylate cyclase activity of a pigeon erythrocyte lysate starts to increase immediately upon the addition of peptide A_1 and continues to increase at a constant rate until a certain maximum activity is reached. The rate of activation is proportional to the A_1 concentration and significant activation can be achieved with less than one copy of A_1 per erythrocyte ghost (Gill, 1976).

Since peptide A_1 has little effect on intact cells, it is supposed that the remainder of the toxin molecule is involved in the insertion of A_1 through the plasma membrane and into the cytosol. This paper is concerned with experiments that relate to the arrangement of, and the interaction between, the various peptides of the toxin. The results suggest an arrangement of subunits in the toxin that, in turn, suggests possible ways in which A_1 may be assisted across a plasma membrane.

Methods

Cholera Toxin. Cholera toxin and choleragenoid were purified by R. A. Finkelstein, Ph.D., The University of

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Texas, Southwestern Medical School, Dallas, Texas (Finkelstein and LoSpalluto, 1970). The toxin was obtained from the National Institute of Allergy and Infectious Diseases through the courtesy of Dr. Carl E. Miller. Choleragenoid was a gift from Dr. Finkelstein. The materials were supplied as powders containing salts and were dissolved in water to give 1 mg/ml of protein in 1 mM EDTA, 3 mM sodium azide, 0.2 M NaCl, 50 mM tris-HCl, pH 7.5.

Polyacrylamide (10%) gel electrophoresis in the presence of 0.1% dodecyl sodium sulfate was conducted according to Weber and Osborn (1969) except that the sample buffer contained no thiol reagent unless otherwise stated and the samples were not heated in the sample buffer unless stated. Cross-linked products to be scanned were separated on longer gels containing half the normal amount of methylenebisacrylamide and ran for 6.5 h at 7.5 mA per gel. All gels were stained for 3 h with 0.1% Coomassie blue. After destaining, gels were scanned at 540 nm using a Gilford Model 2410 linear transport photometer. Positions of bands were determined from the scans.

Molecular weight markers contained in the same or parallel gels included paramyosin, bovine serum albumin, diphtheria toxin and its fragments A and B, catalase, pyruvic kinase, glutamic dehydrogenase, rattlesnake phosphodiesterase, aldolase, aspartic transcarbamylase, carbonic anhydrase, and the fragments of myoglobin described in the text. No very basic proteins were used for calibration since these run slowly. Cholera toxin and choleragenoid both have isoelectric points near neutrality.

Cross-Linking. Samples were dialyzed overnight against 0.2 M triethanolamine-HCl buffer, pH 8.5. To 100 μg of protein (100 μl) were added 1 mg of dimethyl suberimidate dissolved in 100 μl of the same buffer (Davies and Stark, 1970). Reaction (at 20 °C) was stopped by placing the samples on ice after 45, 90, or 180 min: no change was observed after 45 min, suggesting that the reaction was complete in this time. Increasing the reagent concentration from 5 to 20 mg/ml or the protein concentration fivefold affected the outcome only slightly.

Results

Components A and B of Cholera Toxin. By equilibrium centrifugation, the molecular weight of cholera toxin has been estimated at 84 000 daltons (Finkelstein, 1973) and 82 000 daltons (van Heyningen, 1976). The purified toxin gives a single band on electrophoresis in nondenaturing conditions but two major components (A and B) separate in dodecyl sodium sulfate solution (Figure 1c) (Lönnroth and Holmgren, 1973; Finkelstein et al., 1972, 1974; van Heyningen, 1974; Mendez and Lai, 1975). These are immunologically distinct (Ohtomo et al., 1976; Gill and King, 1975) and have different amino-terminal sequences (Jacobs et al., 1974; Kurosky et al., 1975; Lai et al., 1976).

On polyacrylamide gels in the presence of 0.1% dodecyl sodium sulfate, component A has an apparent molecular weight of about 29 000 daltons, determined both from calibration curves and from the coincidence of its band position with that of carbonic anhydrase whose molecular weight is known to be 29 000. After reduction of disulfide groups with mercaptoethanol or dithiothreitol, component A splits into two peptides: A_1 that has an apparent molecular weight of 23 000–24 000 daltons and A_2 that travels with the tracking dye and whose molecular weight we assume to be about 5500. No further change in the behavior of component A is observed after carboxymethylation, heating to 100 °C, exposure to extremes of pH or treatment with 9 M urea.

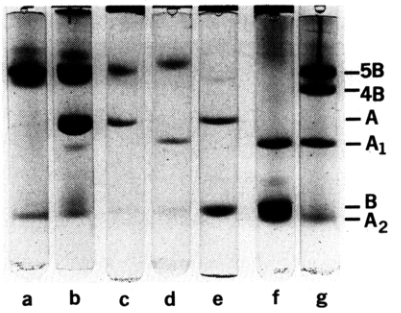

FIGURE 1: Electrophoretic patterns given by choleragenoid (a) and by cholera toxin (b–g). All samples were prepared freshly from powder and mixed with gel sample buffer that contains 0.1% dodecyl sodium sulfate. For gels d and f, the sample buffer also contained 50 mM dithiothreitol. Samples e and f were heated (70 °C, 5 min) in the sample buffer. (a) Choleragenoid, 10 μg; (b) cholera toxin (50 μg)—this overloaded gel shows the diffuseness of B band and minor components before and after B; if the fast-migrating material represents B monomers, it here composes less than 8% of the total component B; (c) 5 μg, (d) 5 μg, reduced; (e) 10 μg, heated, (f) 20 μg, reduced and heated; (g) 20 μg (an aged sample showing partial conversion of pentameric B to tetrameric and monomeric B).

The migration rate of unheated and unreduced component B corresponds to an apparent molecular weight of about 52 000–53 000. After reduction with dithiothreitol, it consistently migrates slightly more slowly with an apparent size of 53 000–54 000 daltons. A slower migration rate is normally taken to mean that the protein can adopt a more extended configuration after reduction of internal disulfide bonds. The higher molecular weight estimate is more likely to be accurate.

The close agreement between the B size estimate from gels (53 000–54 000) and the value of 54 000 twice determined by equilibrium sedimentation (Sattler et al., 1975; van Heyningen, 1976) was somewhat surprising. The B component is an aggregate of subunits which do not separate in dodecyl sodium sulfate and cannot therefore migrate as random coils on gels. It was anticipated that the migration of B on gels might be slow, because of low detergent binding, or fast because of a compact configuration. It seems that these two tendencies probably cancelled each other to a great degree.

In gels stained with Coomassie blue, the intensity of stain in the B band is generally 1.3–1.5 times the intensity of stain in the A band. Since, as will be shown, the two components are present in a 1:1 ratio, per unit mass B must stain only 70–80% as intensely as does A. This may be a reflection of the residual secondary structure that enables component B to remain in the aggregated form in dodecyl sodium sulfate solution.

The B band is unusually diffuse for a protein of its size. When larger samples are run on gels (Figure 1b), it is evident that the diffuseness is primarily at the leading edge of the band. This phenomenon, and the appearance during storage of toxin of a band with an apparent molecular weight of about 44 000 (Figure 1g), will be discussed in the section on iodination.

There is no indication that any of the changes in migration behavior of A or B is due to the action of endogenous proteases. All the changes discussed occur in the presence of inhibitors of proteolytic enzymes. The migration rate of B is not altered if the toxin is first incubated with trypsin (0.1 mg/ml, 10 min, 37 °C, pH 7.5, with or without 50 mM

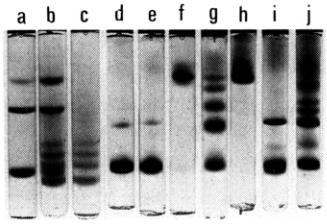

FIGURE 2: (a) Unreduced cholera toxin, B largely disaggregated by heat; (b) mixture of a and c; (c) myoglobin partially cleaved with cyanogen bromide (the four major bands correspond to (from top to bottom) myoglobin (16 950), fragment I plus II (14 450), fragment I plus III (10 700), fragment I (8700). d–j are from a separate experiment: (d) 10 μg choleragenoid, heated; (e) same as d, thiol reduced; (f) same as e, but not heated; (g) choleragenoid cross-linked with dimethyl suberimidate (90 min) and heated, 30 μg; (h) same as g, not heated; (i) cholera toxin, 12 μg, heated; (j) cross-linked cholera toxin, 30 μg, heated.

dithiothreitol). Trypsin does hydrolyze A but the products are all small.

Subunits of B. Component B is composed of subunits that migrate as a single, symmetrical band during gel electrophoresis in 0.1% dodecyl sodium sulfate or in 8 M urea at pH 2.7 or 8.7. Disaggregation of B into its subunits can be effected by heat, by acid, by denaturing agents, or by a combination of these treatments.

A pH of about 3 is required for complete disaggregation at room temperature in the absence of denaturing agents. With 0.03% dodecyl sodium sulfate, a pH of 4 is sufficient and with 0.1% dodecyl sodium sulfate disaggregation is complete at pH 6.2. At pH 6.75, 0.1% dodecyl sodium sulfate, about one-half of component B disaggregates in 30 min. There is even a slight degree of disaggregation at pH 7.5, the normal pH of the sample application buffer used, but this is suppressed if the pH of the application buffer is raised to 9.0. (The toxin experiences pH's up to 9 or 10 during the gel run.)

B also disaggregates when heated in 0.1% dodecyl sodium sulfate. At pH 7.2 about one-half disaggregates in 5 min at 60 °C and all disaggregates in 5 min at 70 °C. Disaggregation by heat is assisted by acid and is slightly reduced by alkaline pH's. Reduction of the disulfide bonds in B by dithiothreitol assists the disaggregation slightly.

The behavior of choleragenoid (Figure 1a), and its disaggregation into subunits, closely parallels that of component B of the complete toxin except in one small respect. Choleragenoid appears to contain a small proportion of its subunits as "stuck" dimers (mol wt about 21 000) that do not disaggregate into monomers even under quite severe conditions (Figure 2d). This may be the material isolated by Finkelstein et al. (1972). Otherwise, the disaggregation of component B appears to be an all-or-none process for aggregates of intermediate size (tetramers, trimers, and dimers: see below) are not generally seen.

The subunits of B generated by heat or acid migrate on gels in the presence of dodecyl sodium sulfate slightly behind the tracking dye (Figure 1) but frequently overlap the A_2 band which runs with dye. The resolution is not substantially improved by varying the composition of the gel, or the running time, or by including 9 M urea in the gel and buffers. By extrapolation from the migration rates of heavier marker proteins, the apparent molecular weight of the B subunits is about 10 500. Values of about 10 600 (reduced) or 10 400 (disulfides intact) are obtained by direct comparison with the migration of cyanogen bromide fragments of myoglobin included in the same gel (Figure 2 a–c). Boiling in 9 M urea does not further change the migration rate.

Both before and after reduction, the apparent mass of a B subunit is almost precisely one-fifth of the apparent mass of the parent aggregate determined from gels or by equilibrium sedimentation, suggesting that the toxin contains five B subunits. However, there are relatively few pentameric proteins, and other workers have interpreted their data for cholera toxin as indicating four (Finkelstein et al., 1972; van Heyningen, 1974; Jacobs et al., 1974), six (Delaney, 1974; Lai et al., 1976), seven (Lönnroth and Holmgren, 1973; Staerk et al., 1974), or eight (Cuatrecasas et al., 1973) B subunits per molecule. Thus it was advisable to confirm the stoichiometry by a different method.

Cross-Linking Experiments. The bifunctional reagent dimethyl suberimidate was used to form covalent cross-links between the subunits of choleragenoid. The solution was then heated to disperse those subunits which had not been covalently connected. On dodecyl sodium sulfate gels, the mixture of products was resolved into five bands (Figure 2g). (Copies of gel scans will be published elsewhere (Gill, 1975b) or will be provided on request.) The slowest band migrated with unheated native B and with unheated cross-linked B, and the fastest band migrated at the same rate as the original B subunits. The banding positions correspond closely to molecular weights in the ratio 1:2:3:4:5 irrespective of the particular molecular weights assigned. There was no material at the position expected for a hexamer of B. It is therefore confirmed that choleragenoid consists of five subunits.

The products formed by reacting whole toxin with dimethyl suberimidate and then heating were incompletely resolved on standard gels (Figure 2j) but on longer gels containing less methylenebisacrylamide they were resolved into nine bands that could be identified as B, 2B, 3B (as in cross-linked choleragenoid) and A, AB, A2B, A3B, A4B, A5B. The A band co-migrated with authentic A; A5B had an apparent molecular weight (82 000) close to the values (82 000 and 84 000) determined for whole cholera toxin by equilibrium sedimentation. The AnB series had apparent molecular weights that fitted closely to the ratios, a, a + b, a + 2b . . . where a = 2.86b, but independent of the actual masses. The best fit to a standard calibration curve established with independent protein markers in the same or parallel gels was obtained if the apparent mass of A was taken as 29 000 and of a B subunit as 10 500. The banding position of A6B, if it had existed, could be predicted from the calibration plot but was vacant. Thus, cholera toxin seems to contain the same five B subunits as choleragenoid, plus a single A subunit that can be cross-linked either to individual or to cross-linked B subunits.

In the cross-linked toxin, the intensities in the B series declined rapidly (B > 2B > 3B; 4B and 5B, if present would be buried beneath other bands). This decline is expected since, the more B subunits that are covalently connected, the more likely it is that the assembly will be attached also to A. Eighty percent of the A subunit was engaged in cross-linking.

In comparison with cross-linked choleragenoid, the cross-linked toxin contained a higher proportion of unconnected B monomers and considerably fewer B pentamers (as A5B). Thus component A must prevent, perhaps sterically, the formation of certain cross-links between B subunits.

A more complex pattern was obtained upon electrophoresis of *reduced* cross-linked toxin. Certain features could,

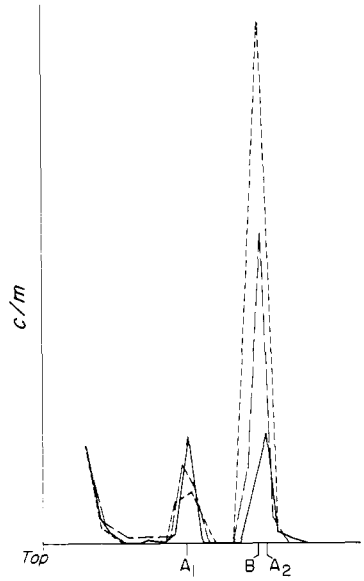

FIGURE 3: Fractionation of [^{14}C]carboxymethyl cholera toxin. Cholera toxin, 4 mg/ml, which had been dialyzed against 0.5% dodecyl sodium sulfate, 1 mM EDTA, 0.2 M Tris-HCl, pH 9, was mixed with 3 mM dithiothreitol under nitrogen. Long dashes: similar sample heated to 80 °C for 2 min. Short dashes: 9 M urea also present, sample heated to 80 °C for 2 min. Reduction was continued for 1 h at room temperature in the dark. [1-^{14}C]Iodoacetamide (2.4 μCi/μmol) was added to a concentration of 6 mM and the incubation was continued. After 30 min, a large excess of mercaptoethanol was added, the control sample was heated to disaggregate component B, and the samples were subjected to electrophoresis on dodecyl sodium sulfate gels. Slices (1 mM) were extracted and counted. The peaks correspond exactly to bands on stained companion gels. The counts near the top of the gels represent carboxymethylated dithiothreitol and carboxymethylated mercaptoethanol.

however, be interpreted unambiguously. A new band appeared at about 17 000 daltons and could only be A_2B. The band coincident with authentic A survived reduction: this must represent A_1 linked to A_2. The slowest band had an apparent size of 82 000 daltons and is therefore likely to be A_1A_25B. The best fit to independent calibration curves was obtained if the apparent sizes of B, A_1, and A_2 were taken as 10 500, 23 500, and 5500, respectively. In this case, all the peaks and shoulders could be assigned to one of the four series B, 2B . . . ; A_1, A_1B . . . ; A_2, A_2B . . . ; and A_1A_2, A_1A_2B . . . . Whether or not the assignments are correct in detail, it was clear that most of the A_2 was cross-linked and most of the A_1 was not. The majority of the A_1 that was combined was linked only to A_2.

Distribution of Sulfhydryl Groups. Cholera toxin contains 12 half-cystine molecules and choleragenoid contains 10 (Delaney, 1974; Finkelstein, 1973 (page 578)). The arrangement of the cysteines was determined by reacting reduced cholera toxin with [^{14}C]iodoacetamide, separating the peptides from each other and from excess reagent by dodecyl sodium sulfate gel electrophoresis, and determining the amount of ^{14}C label associated with the A_1, A_2, and B bands. If the toxin was reduced and carboxymethylated at room temperature in the presence of 0.5% dodecyl sodium sulfate, only two cysteines reacted, one on A_1 and one on A_2. The other ten cysteines were all located on component B but they did not react with iodoacetamide unless the B subunits were separated from each other (by heating) and even then did not react fully unless 9 M urea was also present (Figure 3). Under the most severe conditions (10 mM dithiothreitol, 0.5% sodium dodecyl sulfate, 9 M urea, heated to 80 °C for 2 min, then reacted with 20 mM iodoacetamide), B reacted with approximately ten iodoacetamide molecules. Similar results have been obtained by Lai et al. (1976).

The toxin therefore has one disulfide bridge connecting A_1 and A_2 and one disulfide bridge internal to each of the five B subunits. The latter, although readily reduced (vide the change in migration rate between Figures 1c and 1d), are not able to react with iodoacetamide, even in the presence of 0.5% dodecyl sodium sulfate. Again this suggests a rather strong internal bonding within each B subunit.

Iodination of Cholera Toxin and Formation of B Tetramers. On several occasions cholera toxin was reacted with 0.5–2.5 atoms of ^{125}I per molecule by the chloramine T method and the iodinated species were identified by gel electrophoresis.

In each case most of the iodine reacted with tyrosine residues on peptide A_1. Peptide A_2 and the pentameric form of B both contain tyrosine but do not react. Presumably their tyrosine residues are involved in inter-subunit interactions and are thereby masked.

Iodine label was present in B subunits and in a larger species which was not present before iodination. The larger species was identified as B tetramer by its position on the gel and by its quantitative conversion to B subunits by acid or heat. In the example shown in Figure 4, about one-third of the original B was present as the tetramer. Component B must be arranged in such a way that tyrosines are masked in the pentamer but become exposed on breakdown to tetramer and monomer. As explained in the Discussion, it is most probable that the B pentamer is a closed ring while the tetramer is an open form with exposed faces on the terminal subunits.

Breakdown of pentamers to tetramers and monomers during iodination appears to be merely an accelerated expression of a process that occurs slowly under other conditions. Several toxin solutions have, on storage at 4 °C, shown progressive conversion of 5B to 4B + B over a period of weeks. Gel g of Figure 1 shows an intermediate stage in such a conversion. The natural dissociation can also be inferred from the appearance of the major B band on overloaded gels (gel b in Figure 1). Whereas most proteins give bands with sharp leading edges and diffuse trailing edges, the B aggregate band is more diffuse on its leading edge as if it suffers partial, and readily reversible, disaggregation during the gel run. Presumably iodination, or some other chemical modification that may occur during storage, traps the dissociated species by preventing reassociation.

Re-assembly of Cholera Toxin from Its Subunits. Hitherto functions have been assigned to component B and to peptide A_1 but the role of A_2 has not been established. One approach to this question was to determine whether toxic material could be reconstituted from purified A_1 and B in the absence of A_2. Cholera toxin was fractionated by polyacrylamide gel electrophoresis and gel slices containing individual peptides were placed in dialysis bags in various combinations and dialyzed to remove detergent. The eluates from the gel slices were then tested for toxicity by determining their ability to increase local vascular permeability when injected into the skin of a rabbit. It was possible with this procedure to recombine components A and B to functional cholera toxin albeit in low yield. Per μg, the starting toxin contained about 10 000 skin blueing doses, and the toxin reconstituted from A and B over 200. However, there was no (<2 blueing doses/g) recovery of toxicity if peptide

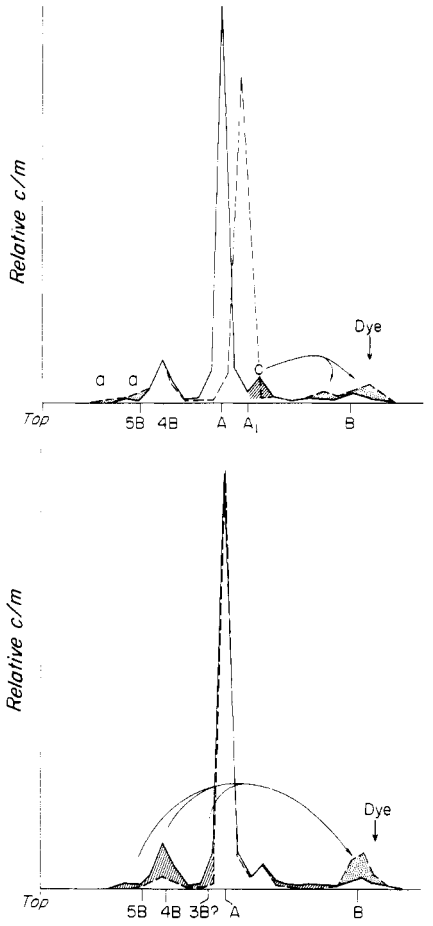

FIGURE 4: Distribution of radioactive material in [^{125}I]toxin fractionated by sodium dodecyl sulfate gel electrophoresis. Cholera toxin, as a freshly prepared solution, was iodinated with two atoms of ^{125}I per 84 000 daltons by the method of Greenwood et al. (1963). Unreacted iodine, 4% of the total, was removed by chromatography on Sephadex G50. The specific activity of the iodinated protein was about 10^5 cpm/μg. Twenty-microgram portions were mixed with a gel sample buffer that includes 0.1% sodium dodecyl sulfate and fractionated by electrophoresis on sodium dodecyl sulfate gels. The gels were stained, photographed, and sliced, and the radioactivity of the slices was measured in a Picker Liquimat β-γ counter. All counts applied to the gel were accounted for. The position of stained bands are marked on the abscissa. A_2 travels with the tracking dye (marked). (Top): Effects of disulfide reduction. The solid line represents a sample with disulfide bonds intact. Sixty-two percent of the counts are associated with component A. The dashed line represents a sample for which 50 mM dithiothreitol was included in the gel sample application buffer. Sixty-two percent of the counts are now associated with A_1. Stippled areas represent material present only after reduction. Aggregates generated by dithiothreitol are marked "a". As these are heat labile and acid labile, they presumably include B subunits. The hatched area, marked C (about 19 000–20 000 daltons), can be seen on overloaded stained gels (e.g., Figure 1b). In the presence of dithiothreitol, it breaks down (arrows) into two species with molecular weights 13 000 and presumably 6000—7000. This material is probably a contaminant representing about 2% of the total protein. (Bottom): Changes effected by heating. The solid line represents unheated cholera toxin, as in panel a. The dashed line represents cholera toxin, which, after mixing with the sample application buffer, was heated at 70 °C for 5 min. The position that a trimer of B would occupy is marked.

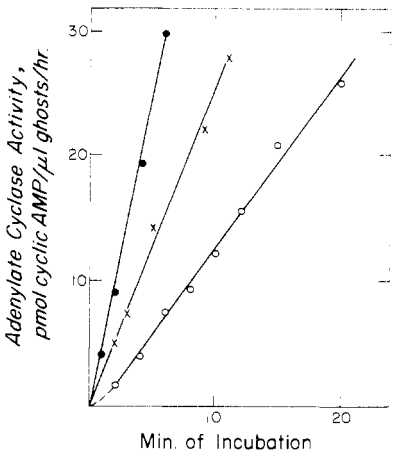

FIGURE 5: Activation of adenylate cyclase in vitro by native toxin, reduced toxin, and reduced and denatured toxin. Pigeon erythrocytes lysed by rapid freezing and thawing in two cell volumes of 0.13 M NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (Hepes), pH 7.3, were supplemented with 10^{-4} M NAD and 5×10^{-3} M ATP and placed in a water bath at 26 °C. At zero time, one-fiftieth volume of cholera toxin at 50 μg/ml was added. Samples containing the equivalent of 10 μl of packed cells were removed at intervals and rapidly diluted with 1.5 ml of ice-cold saline. The ghosts were recovered by centrifugation and their adenylate cyclase activities immediately measured by the method described elsewhere (Gill, 1976): (○) control toxin; (X) toxin preincubated 10 min 37 °C with 1 mM dithiothreitol; (●) toxin preincubated 10 min 37 °C with 1 mM dithiothreitol and 0.5% dodecyl sodium sulfate. In this case, 0.01% dodecyl sodium sulfate was present during the incubation with erythrocyte lysate.

A_2 was omitted, that is, if A_1 and B were mixed. Thus A_2 is not vestigial but is clearly necessary for the assembly of toxic material. It seems likely to be an adaptor piece that allows A_1 to associate in the proper configuration with the B assembly.

Effect of Disruption of Cholera Toxin on Its Activity in Vitro. The intracellular action of cholera toxin may be conveniently assayed by incubating the toxin with pigeon erythrocyte ghosts, NAD, ATP, a protein present in erythrocyte cell supernatant, and a thiol reagent. The activity of the adenylate cyclase carried by the ghosts rises during the incubation until it reaches a certain maximum level that may represent the point at which all copies of the enzymes have been modified (Gill and King, 1975; Gill, 1976).

Pre-treatment of the toxin with dithiothreitol (1) abolishes the later thiol requirement, (2) abolishes a lag of about 1 min that otherwise exists before the rate of cyclase activation reaches its maximal level, and (3) approximately doubles the rate at which the cyclase is activated (Figure 5). These effects presumably result from the reduction of the disulfide bond between A_1 and A_2. Carboxymethylation of the toxin does not alter the activity further, showing that the importance of the reduction of the toxin lies in the cleavage of the sole covalent bond between A_1 and A_2 rather than in the generation of sulfhydryl groups.

An additional increase in activity is obtained by pre-incubating the reduced toxin in dodecyl sodium sulfate solution (Figure 5). A similar degree of stimulation is obtained if isolated subunit A is pre-incubated in dodecyl sodium sulfate. Thus the stimulation observed with the complete toxin can be attributed to the separation of the active peptide, A_1, from noncovalent linkage to peptide A_2 rather than from component B.

It follows from these results that the complete toxin has little or no enzymic activity but must be first re-organized in such a way that its peptide A_1 is liberated as an independent species.

Discussion

The results presented show that cholera toxin consists of a single A subunit of 29 000 daltons and five B subunits of about 10 600 daltons each. The internal interactions within

each B subunit appear to be unusually strong. Although reduced B subunits readily separate from each other when heated in dodecyl sodium sulfate, it is necessary to heat them also in 9 M urea before all their cysteine residues are available for carboxymethylation. Subunit A readily separates from the B pentamer (choleragenoid) in dodecyl sodium sulfate. On reduction, A is further split into peptide A_1 (apparent size ca. 23 500 daltons) that is active in vitro and peptide A_2 (ca. 5500 daltons). The cross-linking reagent dimethyl suberimidate forms covalent bonds between individual B's, between A_1 and A_2, and between a B subunit and A_1 or A_2 or possibly both. Although A_1 is close enough to occasionally form a cross-link to a B subunit, its most important contacts are with peptide A_2, through a single disulfide bond and through noncovalent attachments that must be broken before A_1 exhibits its maximum activity in vitro. Reconstitution experiments show that A_2 is necessary for the proper association of A_1 and B and it therefore most probably lies between A_1 and B. A_2 is readily cross-linked both to A_1 and to B.

These data suggest the following arrangement of subunits. The five B subunits probably form a closed ring for any other arrangement involves nonequivalent subunit–subunit interactions. The single A subunit is presumably located more or less on the axis of the ring although, being itself asymmetric, it must be arranged asymmetrically. A_2 lies proximal to the ring with respect to A_1 and, since the B ring almost inevitably has a central hole, A_2 may extend some distance between the B's. In further conformation of this arrangement, Sattler et al. (1975) were able to separate reduced cholera toxin into (ganglioside binding) $A_2$5B and free A_1.

The proposed arrangement is similar to that deduced by Ohtomo et al. (1976) from electron microscopic examination of negatively stained toxin. These workers found a number of compact (ca. 80–90 Å) structures, many of which appear as rings with a central core. The core is absent from choleragenoid. This model provides explanations for several other observations.

1. In acid solution in the presence of dodecyl sodium sulfate, component B disaggregates from pentamers to monomers without forming significant amounts of stable tetramers, trimers, or dimers. Presumably, removal of one subunit in acid facilitates the disaggregation of the remaining four since a ring could be more stable than an open form.

2. Subunit A restricts the number of cross-links formed by dimethyl suberimidate between B subunits. The prohibited cross-links probably include those across the central hole; for example, whereas A3B formed by cross-linking toxin gives a sharp symmetrical band, 3B formed by cross-linking choleragenoid gives a broad and slightly asymmetric band, suggesting that it contains both linear trimers involving tangential cross-linking and triangles involving cross-links across the center. The two types must have different shapes, even when heated in detergent, and travel at slightly different rates during electrophoresis.

3. Tetramers of B are sometimes formed when toxin solutions are stored at 4 °C (e.g., Figure 1g) or are reacted with iodine (Figure 4). tyrosine residues on the tetramers, but not on the parent pentamers, react with iodine. Presumably tyrosine residues are present on those surfaces of the B subunits that are normally engaged in intersubunit binding and these tyrosines become exposed on the terminal subunits when one subunit leaves the ring.

This model of the structure of the toxin may suggest how the protein functions.

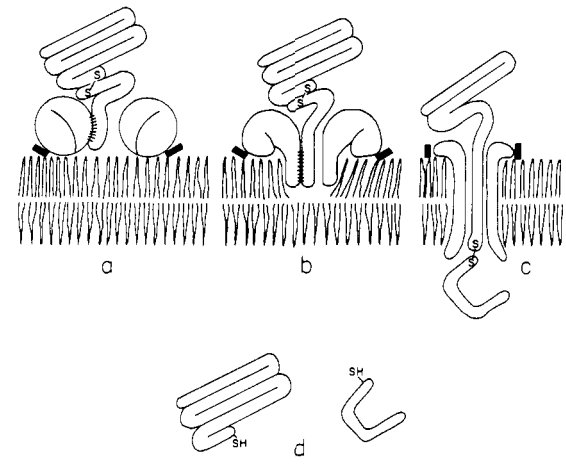

FIGURE 6: One possible mode of entry of peptide A_1. (a) Hatching represents noncovalent bonds between A_2 and B. It is suggested that the initial binding of toxin to surface receptors (shaded) may be followed (b) by a change in the conformations of the B subunits which enter the membrane, bind to lipids, and create a hydrophilic channel. (c) The modified B no longer binds to A_2. Component A, now free to diffuse, may sometimes diffuse inward, unfolding on the outside and spontaneously refolding inside the cell. B remains as a membrane protein. (d) The disulfide bond between A_1 and A_2 is reduced by intracellular glutathione.

Recent evidence suggests that peptide A_1 acts enzymically while free to diffuse through the cytosol of intoxicated cells. It is thus necessary to consider by what means A_1 crosses the plasma membrane (or the membrane of a vesicle if pinocytosed). It has been shown here that the full activity of A_1 is realized only when A_1 is separated from A_2 and B by reduction and denaturation. Presumably such separation occurs in vivo also. It is most reasonable to suppose that the intoxication of an intact cell involves the insertion into the cell of A_1, and perhaps A_2, but not of B which remains on or in the membrane (Peterson et al., 1972; Gill and King, 1975). The interaction of the toxin with the cell membrane, which is primarily the function of component B, may be followed by some conformational re-arrangement of the toxin that weakens the A–B interactions and allows A_1 to enter.

There is ample evidence that cholera toxin binds to some molecule common to vertebrate cell surfaces and there are strong indications that the receptor may be ganglioside G_{M1} or a related molecule. G_{M1} binds to component B in the aggregate or as subunits (van Heyningen, 1974; Holmgren and Lönnroth, 1975). Presumably a toxin molecule first binds to a cell when one B subunit binds one molecule of ganglioside (or similar molecule). By diffusion in the plane of the membrane, other receptors become correctly positioned and all five B subunits are eventually bound. The toxin is then held flat against the membrane (Figure 6a). The result of such binding is a local increase in the concentration of toxin so that the subsequent penetration of A_1 (by whatever means) is more likely.

It is possible that component B has no further role in the process and that A_1 then crosses the membrane unaided. A second possibility is illustrated in Figure 6. Here account is taken of the ring structure of the toxin and of the strong internal hydrophobic interactions noted for the B subunits. It is possible that these internal bonds are replaced by protein–lipid interactions so that the ring of B subunits at the same time unfolds and enters the plasma membrane, retaining the central hole, and eventually forms a hydrophilic channel

through which component A can diffuse. There is no obvious reason why such a change in conformation need be energetically unfavorable. However, the activation energy for the initiation of the process is likely to be considerable and entry could be slow. Experimentally, the entry of A_1 appears slow for no effects attributable to intracellular A_1 have been detected for 15–120 min after the initial binding of toxin to intact cells.

The disulfide bond in subunit A is presumably reduced when the subunit first encounters intracellular glutathione. There is at present no clear basis for deciding whether this occurs at the inner face of the plasma membrane or (as depicted in Figure 6) whether the entire component A enters the cell and is then reduced. In either case, the intracellular environment may also be conducive to weakening noncovalent interactions between A_1 and A_2.

Whether A_1 crosses the membrane by itself, through a channel created by component B, or through a preexisting channel, it is likely that it must cross in an unfolded configuration and refold when inside the cell. Such refolding presents no difficulty for A_1 readily renatures. If heated to 100 °C in dodecyl sodium sulfate solution, A_1 displays its full activity as soon as it is cooled again (Gill, D. M., unpublished observation).

It is not necessary to postulate any coupling of the entry of A_1 to an energy-generating process for it now seems that the entry process may be sufficiently rare to occur by diffusion only. It is possible to raise the adenylate cyclase activity of lysed pigeon erythrocytes about tenfold in 30 min using only one A_1 molecule per ghost. This is a more rapid activation than can be achieved with intact erythrocytes using any amount of toxin, even though many hundred toxin molecules bind to the outside of each cell (King et al., 1976). Thus, in this case at least, the insertion of A_1 into the cytoplasm appears to be an inefficient process, with most of the bound A_1 remaining at the surface or perhaps diffusing in the wrong direction, into the extracellular medium.

There remain unanswered, and certainly interesting, questions concerning the genesis of cholera toxin. A_1 and A_2 could be products of a single gene coding for a precursor protein that is subsequently cleaved or nicked. Further work will be necessary to test for the putative precursor and to determine whether it is processed before or after secretion from the parent bacterium. It is less likely that the entire toxin molecule is derived from a single precursor protein that is subsequently cleaved into seven peptides, of which five are identical. The more reasonable possibility is that there are separate genes for A and for B and that B is transcribed or translated faster than A.

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