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Structure and Activity of Diphtheria Toxin

II. ATTACK BY TRYPSIN AT A SPECIFIC SITE WITHIN THE INTACT TOXIN MOLECULE*

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SUMMARY

Most preparations of diphtheria toxin that we have examined consist predominantly of mixtures in various proportions of two proteins, each of molecular weight about 63,000. As judged by their electrophoretic behavior on polyacrylamide gels run in the presence of sodium dodecyl sulfate, one of these consists of intact 63,000-dalton polypeptide chains, while the other is composed of two fragments of molecular weight 24,000 and 39,000 (Fragments A and B, respectively) linked by at least one disulfide bridge. Here we report that when these preparations are treated at 25° with low concentrations of trypsin (0.25 to 1.0 μg per ml), a single scission is introduced at a specific site in the former (intact toxin) converting it into a protein apparently identical with the latter (nicked toxin). Thus nicked toxin in the original preparations must be a derivative of intact toxin which arose by attack of proteases from the bacterial culture in which the toxin was produced.

Fragment A contains 1 half-cystine residue, and is therefore linked to Fragment B in nicked toxin by one disulfide bridge. Fragment A is stable to heating at 100°, and exposure to pH values from 2 through 12. Fragment B is relatively unstable, and precipitates even at neutral pH.

We have shown previously that toxin is almost devoid of enzymic activity unless treated with thiols. The activity is probably entirely due to Fragment A released upon dissociation of nicked toxin in the presence of thiols. When toxin is treated with trypsin the intact chains are converted into a form which can be dissociated by thiols into fragments apparently identical with A and B. Hence the specific activity of the preparation is enhanced by trypsin treatment because of the greater amount of Fragment A released by thiols.

The toxicity of toxin is not significantly changed by treatment with trypsin, which indicates that both intact and nicked toxin are toxic. Fragment A alone is not toxic. Linkage to Fragment B is apparently required for toxicity, perhaps to facilitate entry of Fragment A into cells. Treatment of toxin with thiols decreases the toxicity, presumably as a result of dissociation of the nicked fraction into nontoxic Fragments A and B.

Diphtheria toxin is known to inactivate the peptidyl transfer RNA translocation factor from eukaryotic cells, transferase II, by catalyzing transfer of the adenosine diphosphate ribose moiety of NAD^+ into covalent linkage with the factor (1, 2). In the preceding article it was shown that the toxin is almost completely lacking in activity in this reaction unless it has been previously treated with thiol or thiol is included in the assay mixture (3). Treatment with thiol was shown to activate toxin by dissociating a fraction of the 63,000-dalton toxin protein into an enzymically active 24,000-dalton fragment (Fragment A) and a 39,000-dalton fragment (Fragment B) presumed to be inactive. We concluded that free Fragment A is probably entirely responsible for the catalytic activity of thiol-treated toxin. The trace of activity of untreated toxin may have been due to contamination by free Fragment A, which has been found in substantial quantity in crude preparations of toxin.

The dissociable fraction of toxin, which apparently consists of molecules containing one A and one B fragment linked by at least one disulfide bridge, constitutes a variable proportion of the total protein in different preparations (from 0 to almost 100%) (3). The remaining protein was judged by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate to consist predominantly of single, intact chains of molecular weight 63,000, which were catalytically inactive either before or after exposure to thiols. Our inability to separate the intact chains from the dissociable fraction of toxin by any of a number of methods led us to speculate that they might consist of related proteins.

Here we report that in the presence of low concentrations of trypsin the intact 63,000-dalton chains (intact toxin) are cleaved at a specific site, converting them into molecules similar to or identical with those of the dissociable fraction (nicked toxin). After trypsin treatment, virtually 100% of the toxin can be dissociated by thiols into fragments apparently identical with A and B. The specific activity of the preparation in catalyzing the ADP ribosylation of transferase II is thus increased by virtue of the greater amount of active Fragment A released in the

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presence of thiols. These results imply that the dissociable fraction in our original preparations is actually a derivative which apparently arose through the action of a protease or proteases on intact toxin, either in the bacterial culture from which the toxin was derived, or during isolation and purification of toxin. This in turn indicates that the toxin must be synthesized as a single polypeptide chain of molecular weight 63,000, or as part of a larger chain. A preliminary report of this work has appeared (4).

EXPERIMENTAL PROCEDURES

Reagents—Pronase was purchased from Calbiochem; fluorescein mercuric acetate from Sigma; L-1-toluenesulfonylamido-2-phenylethyl chloromethyl ketone-treated trypsin, chymotrypsin, and soy bean trypsin inhibitor were products of Worthington. ^{14}C-Iodoacetamide (3.16 mCi per mmole) was obtained from New England Nuclear, ^{14}C-chloromercuribenzoate (12.0 mCi per mmole) and ^{14}C-leucine (320 mCi per mmole) were from Schwarz BioResearch. Subtilisin was a gift from Dr. A. Glazer, Department of Biological Chemistry, UCLA School of Medicine. All other materials were obtained as previously described (3).

Diphtheria Toxin—Purified toxin, lot 007, was prepared as described previously (3).

Fragment A—Approximately 400 mg of toxin in 30 ml of buffer (50 mM Tris-HCl, pH 8.2-1 mM EDTA) was incubated for 30 min at 25° in the presence of 1 μg per ml of trypsin. Dithiothreitol was then added to a final concentration of 0.1 M and the mixture was further incubated for 90 min at 37°. A precipitate containing Fragment B and some toxin formed and was removed by centrifugation. The remaining solution was dialyzed overnight at room temperature against 50 volumes of 0.1 M 2-hydroxyethyl disulfide in buffer. This prevented dimerization of A by formation of mixed disulfides with the free sulfhydryl group of A (5). The protein was then purified by chromatography on a column of Sephadex G-100 (5 × 86 cm) equilibrated with 50 mM Tris-HCl, pH 8.2-1 mM EDTA. About 120 mg of purified protein were recovered. Unless indicated otherwise, dilutions of toxin or Fragment A were in 50 mM Tris-HCl, pH 8.2, 1 mM EDTA, and 50 μg per ml of crystalline bovine serum albumin (Buffer D).

Assay of ADP Ribosylation Activity—The enzymic activity of toxin or derivatives of toxin was assayed by measuring incorporation of radioactive label from ^{14}C-NAD$^+$ labeled in the adenosine moiety into trichloracetic acid-precipitable material in the presence of a partially purified preparation of transferase II. The materials employed and conditions of assay were identical with those described previously (3).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—The gels used contained 10% acrylamide and 0.27% methylenebisacrylamide, in phosphate buffer, 0.1% in sodium dodecyl sulfate. The methods of preparation of gels and samples and the conditions for electrophoresis were as described in the preceding paper (3).

Trypsin Treatment—Unless indicated otherwise, trypsin treatment was carried out by the following procedure. Toxin (300 to 500 μg per ml) in 0.01 M sodium phosphate, pH 7.0, was incubated at 25° for 30 min with 0.4 μg per ml of trypsin. Soy bean trypsin inhibitor was then added to 0.6 μg per ml, and the mixture was incubated several minutes more at 25°.

Protein Synthesis in HeLa Cells—HeLa cells, strain S-3, were grown in spinner culture in Eagle's spinner medium. For measurement of protein synthesis in the culture, 1.25 μCi of ^{14}C-leucine (320 mCi per mmole) were added to a 20-ml portion of culture containing approximately 3 × 10^5 cells per ml, and the suspension was incubated in stoppered 50-ml Erlenmeyer flasks at 37° in a shaking water bath. At 2-hour intervals, 1-ml samples were removed and diluted into 4 ml of spinner medium without calf serum. The cells were then filtered onto Millipore filters, which were washed with four 5-ml portions of 5% trichloracetic acid, dried, and counted.

Amino Acid Analysis—Fragment A (0.03 μmole per sample) was hydrolyzed at 110° in 6 N HCl in sealed, evacuated tubes for periods of 24, 48, and 72 hours. Cystine was determined as cysteic acid according to the method of Moore (6). Analyses were performed on a Beckman model 116 amino acid analyzer.

Treatment of Fragment A with Chloromercuribenzoate—Fragment A (0.086 μmole) was reduced by incubation for 1 hour at 25° in 100 μl of 0.01 M sodium phosphate, pH 7.5, containing 0.1 M dithiothreitol. A sample of 50 μl was removed, mixed with 0.1 ml of 0.1 M ^{14}C-chloromercuribenzoate (0.01 mCi per mmole) and further incubated for 1 hour at 30°. Glycerol was then added to 5%, and the mixture was chromatographed on a Sephadex G-25 column according to the procedure of Erwin and Pederson (7). The radioactivity and ADP ribosylation activity of the column fractions were assayed.

Treatment of Fragment A with Iodoacetamide—The method was a modification of that of Haber and Anfinsen (8). Fragment A (0.24 μmole) in 50 mM Tris-HCl, pH 8.2-1 mM EDTA was incubated for 1 hour with 0.1 M DTT[1] in a volume of 0.1 ml. Portions of 50 μl were then removed, diluted with 0.2 ml of Tris-EDTA buffer, and combined with 0.2-ml samples of 0.26 M ^{14}C-1-iodoacetamide (0.022 mCi per mmole). The mixtures were incubated in the dark at 25°. At intervals up to 2 hours, 100 μmoles of mercaptoethanol were added, and the samples were assayed for ADP ribosylation activity or precipitated with 10% trichloracetic acid, filtered, and counted. Guanidine (4 M) was added to some samples before incubation with iodoacetamide, but this was found not to change the amount of radioactivity incorporated.

Disulfide Content of Toxin—The number of disulfide bridges in toxin was determined by measurement of quenching of the fluorescence of fluorescein mercuric acetate as described by Karush, Klinman, and Marks (9). Toxin (0.002 μmole) was incubated with 1 × 10^6 M fluorescein mercuric acetate in 1 N NaOH in a final volume of 10 ml. After 15 min, the fluorescence (excitation 499 nm, emission 520 nm) was read and the disulfide content was calculated from a calibration curve prepared with ribonuclease A which has 4 disulfide bridges.

RESULTS

Almost all the experiments in this as well as the preceding article were conducted using a single preparation of purified diphtheria toxin (lot 007) produced in our laboratory. Approximately 20% of the protein of this lot was dissociable by thiols into Fragments A and B.

Effect of Proteases on Enzymic Activity of Toxin

The specific activity of toxin Preparation 007 in catalyzing the ADP ribosylation of transferase II under standard conditions

[1] The abbreviations used are: DTT, dithiothreitol; SDS gels, polyacrylamide gels containing 0.1% sodium dodecyl sulfate.

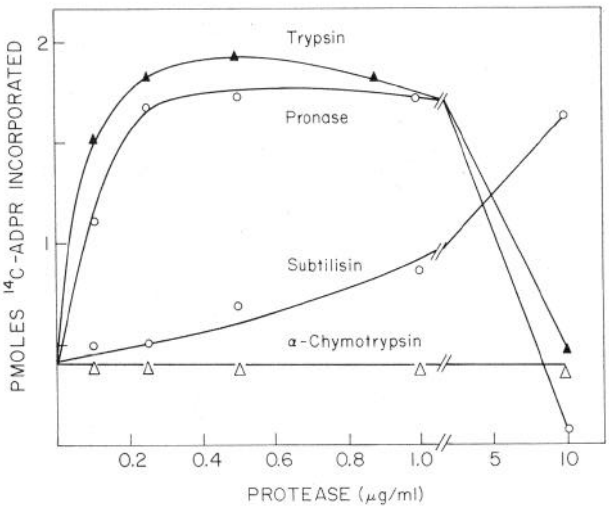

FIG. 1. Effect of proteases on the enzymic activity of toxin. Toxin, lot 007 (0.17 mg per ml, in 50 mM Tris-HCl, pH 8.2-1 mM EDTA), was incubated for 30 min at 25° with various proteases as indicated. When subtilisin was used, the buffer contained 1 mM $CaCl_2$ in place of EDTA. After incubation, samples were diluted 1:300 in Buffer D, and 10-μl portions were assayed for ADP ribosylation activity as described previously (3). The assay mixtures contained 40 mM DTT. *ADPR*, adenosine diphosphate ribose.

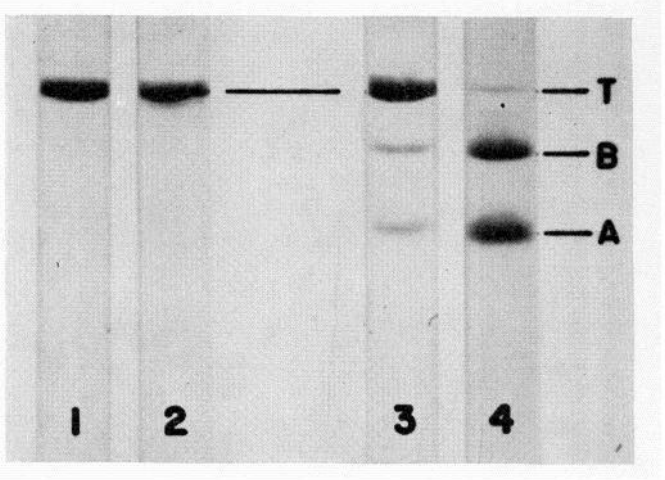

FIG. 2. Electrophoresis of trypsin-treated and untreated toxin on SDS gels. Identical samples of toxin, lot 007 (1.7 mg per ml in 50 mM Tris-HCl, pH 8.2-1 mM EDTA), were incubated for 45 min in the presence or absence of 1 μg per ml of trypsin. Duplicate portions of each sample were then treated at 100° in 1% sodium dodecyl sulfate either with or without mercaptoethanol, and 10 μg of each were electrophoresed on an SDS gel. The gels show samples treated (*1*) without trypsin or thiol, (*2*) with trypsin only, (*3*) with thiol only, or (*4*) with both trypsin and thiol.

was found to be markedly enhanced after incubation with certain proteases. As shown in Fig. 1, after treatment for 30 min at 25° with trypsin or Pronase at 0.25 to 1.0 μg per ml, the toxin was four to five times as active as the control. At 10 μg per ml, trypsin produced no change while Pronase produced a decline in the activity. Subtilisin also activated the toxin but approximately 100-fold higher concentrations of this protease than trypsin were required for equivalent activation. α-Chymotrypsin produced no enhancement up through 10 μg per ml. Treatment of purified Fragment A with trypsin (0 to 10 μg per ml) caused no change in its activity.

The trypsin-treated toxin employed in the experiments below had been treated with trypsin at 0.4 to 1.0 μg per ml for 30 to 45 min at 25°. If it was not to be used immediately, soy bean trypsin inhibitor was added to 1.5 times the concentration of trypsin to prevent further proteolytic action.

Thiol-dependence of Activity of Trypsin-treated Toxin

When the activity of trypsin-treated toxin was measured as a function of the concentration of dithiothreitol in the assay mixture, the curve obtained was identical with that of control toxin, after adjustment for the difference in specific activity of the two preparations (see Fig. 2 of the preceding article (3)). The activity increased up to 20 mM DTT, was maximal between 30 and 50 mM, and declined at higher concentrations of the thiol. The trace of activity of untreated preparations in the absence of thiol was not increased by trypsin, which is consistent with our view that this activity is due to contamination by unassociated Fragment A.

Electrophoresis of Trypsin-treated Toxin in SDS Gels

Trypsin-treated toxin, like untreated toxin, migrated as a single band of molecular weight about 63,000, when run on SDS gels in the absence of thiol (Fig. 2). However, when treated with β-mercaptoethanol prior to electrophoresis, almost the entire preparation was found to dissociate into fragments, in contrast to control toxin in which only about 20% of the protein could be dissociated.

The products of dissociation of trypsin-treated toxin migrated identically with Fragments A and B from control toxin. When trypsin-treated and control toxin were each treated with sodium dodecyl sulfate and mercaptoethanol at 100°, and subsequently mixed and run on SDS gels, no evidence of splitting of either the A or B band was observed.

No evidence of fragments other than A and B was detected in an experiment in which the kinetics of cleavage by trypsin were followed. Toxin was treated with trypsin (0.1 μg per ml) at 25°, and samples were removed at frequent intervals for assay of activity and analysis of band patterns on SDS gels. The increase in specific activity, which reached a maximum after 40 min, was accompanied by a proportional increase in intensity of *Bands A* and *B* and a decline in *Band T*. The specific activity and the band pattern were unchanged from 40 through at least 180 min of trypsin treatment. No trace of other bands was detected at any time during the experiment.

These results indicated, as had been suspected, that the intact and dissociable fractions of toxin were related proteins. Treatment with trypsin apparently introduced a single nick in the intact 63,000-dalton chains which constituted approximately 80% of the protein of this preparation. Dissociation by thiols then yielded approximately four to five times as much active Fragment A, and hence the specific activity of the preparation increased by the same factor.

For the sake of simplicity we assume in the discussion which follows that Fragments A and B derived from trypsin-treated toxin are identical with the fragments from the nicked fraction of control preparations. Because of our inability to differentiate between these on SDS gels, any differences in length which may exist must amount to no more than about 10 amino acid residues.

Fractionation of Trypsin-treated Toxin by Gel Filtration

Trypsin-treated toxin was chromatographed on a column of Sephadex G-100 (superfine) identical with those used in Fig. 5 of the preceding article (Fig. 3*A*). The toxin emerged as a single peak with the ADP ribosylation activity coincident, at the same position as untreated toxin (34 ml; *cf.* Fig. 5 in Reference 3). When trypsin-treated toxin was incubated in 100 mM DTT for 1 hour at 25° prior to chromatography, very little protein or activity remained in the region of 34 ml (Fig. 3*B*). As in the case of thiol-treated control toxin (not treated with trypsin), the major portion of the activity was associated with a peak of protein at 44 ml, which was shown on SDS gels to contain Fragment A together with small amounts of Fragment B. This material was highly active in the absence as well as the presence of 40 mM DTT.

A larger peak of protein at 37 to 38 ml was found to contain mostly Fragment B. However, SDS gels run on samples from fractions in this region in the presence of mercaptoethanol showed that Fragment A was present at concentrations sufficient to account for the activity observed. SDS gels run in the absence of thiol showed that Fragment A was present both in the form of dimer and 63,000-dalton complex with Fragment B. These may have formed from free Fragments A and B through autoxidation, either during passage through the column or in the tubes after collection. Alternatively the reduced toxin may have been incompletely dissociated before application to the column, and further dissociation may have occurred during chromatography, followed by autoxidation in the collection tubes.

The leading edge of the larger peak of protein in the region of 34 ml contained a small amount of intact 63,000-dalton chains plus nicked chains which apparently escaped reduction.

These results are consistent with the idea that the increased amount of Fragment A released from trypsin-treated toxin in the presence of thiols is responsible for the higher specific activity of the preparation as compared with control toxin.

Effect of Trypsin on Other Preparations of Toxin

Other preparations of toxin produced in this laboratory and several from other laboratories have been found to contain intact and nicked toxin in various proportions (3). After treatment with trypsin as described under "Experimental Procedures," all the preparations dissociated completely or almost completely into A and B fragments in the presence of sodium dodecyl sulfate and mercaptoethanol, and the specific activity was found to increase in those and only those preparations which originally contained intact 63,000-dalton chains.

Toxicity of Derivatives of Toxin

Toxicity was determined by assay of activity in inhibiting protein synthesis in cultures of HeLa cells, and the results were confirmed by measurement of lethal activity in guinea pigs. The effect on protein synthesis was measured by following the kinetics of incorporation of ^{14}C-leucine into protein in suspensions of HeLa cells over an 8 to 10-hour period in the presence of various concentrations of toxin or derivative. The results of determinations of the minimum lethal dose for guinea pigs were in agreement with those obtained by this method, but the numbers of animals used in these tests were not sufficient to obtain accurate values of the toxicity.

a. Fragment A—Derived from trypsin-treated toxin it was found to have no effect on protein synthesis in HeLa cells for at least 8 hours at concentrations up through 4 μM; this is approximately 200 times the saturating molar concentration of toxin, which affects protein synthesis in this system within 3 to 4 hours after addition. Doses of 2 nmoles of Fragment A produced no toxemic symptoms in 250-g guinea pigs, over a period of several weeks, while only about 1 pmole of toxin causes death of such an animal in about 4 days. Similar results have been

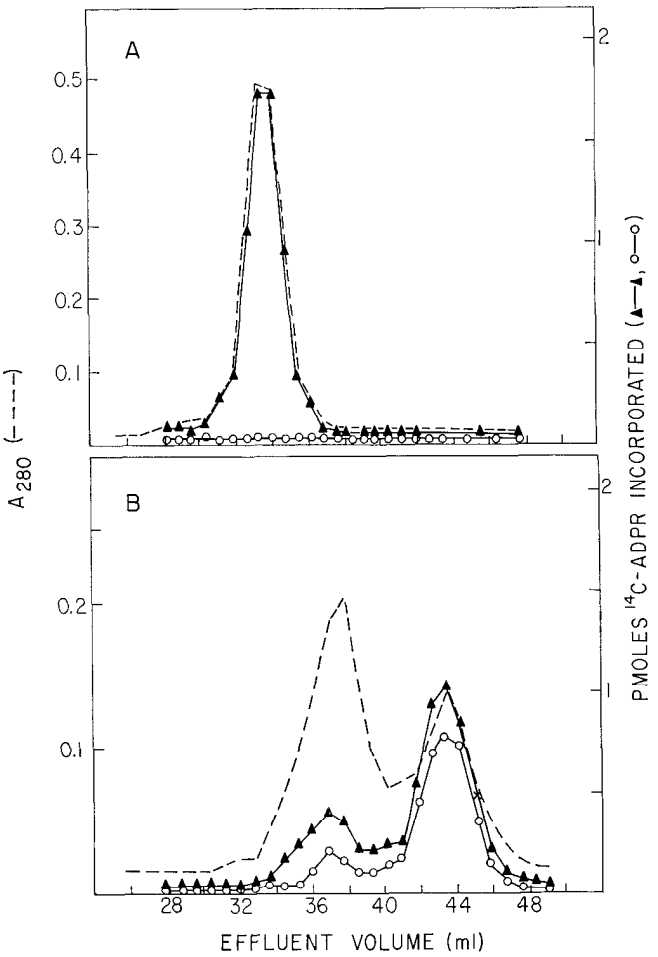

FIG. 3. Chromatography of thiol-treated and untreated trypsin-activated toxin on Sephadex G-100 (superfine). Duplicate samples of toxin were treated with trypsin as described under Fig. 2. At the end of the incubation soy bean trypsin inhibitor was added to a final concentration of 1.5 μg per ml, and after several minutes, DTT (final concentration 0.1 M) was added to one of the samples. After an additional hour at 25°, glycerol was added to both samples to 5%, and each (1.6 mg of toxin in 255 μl) was layered on a column of Sephadex G-100 (superfine) (1.4 × 49.5 cm) equilibrated with 50 mM Tris-HCl, pH 8.2-1 mM EDTA. Chromatography was carried out at 4°, at a flow rate of 2.3 ml per hour. The protein content of each fraction was estimated by measuring the absorbance at 280 nm (- - -). A portion of each fraction was then diluted by a factor of 1500 in Buffer D, and duplicate 25-μl samples were assayed for ADP ribosylation activity either in the presence (▲——▲) or absence (○——○) of 40 mM DTT. *A*, toxin not exposed to thiol; *B*, DTT-treated. Samples from selected fractions were treated with sodium dodecyl sulfate in the presence or absence of mercaptoethanol, and run on gels. The recovery of protein was 98% from Column A and 90% from B. The recovery of activity was 98% from A and 80% from B. *ADPR*, adenosine diphosphate ribose.

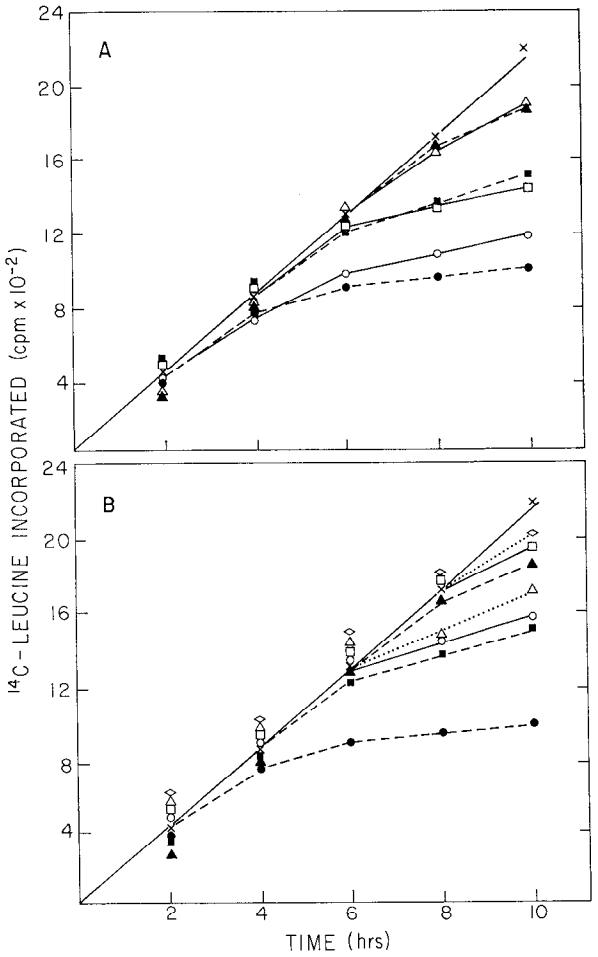

FIG. 4. Kinetics of inhibition of protein synthesis in HeLa cells by toxin or derivative of toxin. Suspensions of HeLa cells (20-ml portions at 3 × 10^5 cells per ml) were incubated with ^{14}C-leucine at 37° in a shaking water bath, and 1-ml portions were withdrawn periodically for determination of incorporated radioactivity. The toxin or derivative thereof was added immediately before the radioactive leucine. *A* shows a comparison of the effects of untreated (——) with trypsin-treated (- - -) toxin at three concentrations (*circles*, 30 nM; *squares*, 8 nM; and *triangles*, 2 nM). Control without toxin (×——×). Trypsin-treated toxin (200 μg per ml in 10 mM sodium phosphate buffer, pH 7.0) had been incubated with 0.4 μg per ml of trypsin for 1 hour at 25°. Soy bean trypsin inhibitor was then added to 0.6 μg per ml, and incubation was continued for a few minutes at 25°. *B* shows the effect of exposure of control or trypsin-treated toxin to 0.1 M DTT for 1 hour at 25°. Trypsin-treated toxin: 30 nM, ●- - -●; 30 nM, treated with DTT, ○——○; 8 nM, ■- - -■; 8 nM, treated with DTT, □——□; 2 nM, ▲- - -▲. Control toxin: treated with DTT, 8 nM, △····△, or 2 nM, ◇····◇.

obtained in less extensive experiments conducted with Fragment A derived from toxin which had not been exposed to trypsin. We conclude that Fragment A is nontoxic.

b. Trypsin-treated Toxin—This toxin (lot 007) has approximately the same specific toxicity as untreated toxin, in contrast to the 4- to 5-fold difference in specific activity between the two in the ADP ribosylation assay. As shown in Fig. 4*A*, at final concentrations of 2 nM or 8 nM, trypsin-treated toxin inhibits protein synthesis in HeLa cells with kinetics identical with those of untreated toxin. At a concentration of 30 nM, trypsin-treated toxin appears to have slightly greater activity than untreated toxin. The basis of this difference is being investigated. No significant difference between the toxicity of trypsin-treated and control toxin has been observed in guinea pig lethality tests.

c. Thiol-treated Toxin—This toxin (lot 007) exhibited slightly lower activity in inhibiting protein synthesis than untreated toxin (Fig. 4*B*). The thiol-treated toxin had been incubated with 100 mM DTT for 1 hour at 25°. The final concentration of DTT in the culture was 0.5 mM, which alone caused no change in leucine incorporation over the period of the experiment.

A much more marked decline in toxicity occurred when trypsin-treated toxin was exposed to DTT under the same conditions. As shown in Fig. 4*B*, after incubation with DTT, the activity of either 30 nM or 8 nM trypsin-treated toxin was decreased by a factor greater than 4. A similar decline in toxicity was detected by guinea pig lethality tests. Toxin which had not been exposed to trypsin showed a slight decline in toxicity for guinea pigs when incubated with DTT.

Properties of Fragments A and B

A future communication from this laboratory will be devoted to a detailed consideration of the physical and chemical properties of Fragments A and B. Here we present certain properties of the fragments which are relevant to the major results of this paper. The fragments employed were from trypsin-treated toxin; it is assumed that these are identical with those from untreated toxin.

Analysis of the amino acid composition of purified Fragment A has revealed that it contains 1.3 half-cystine residues/24,000-dalton protein. Reaction of reduced Fragment A with ^{14}C-labeled chloromercuribenzoate gave a figure of 1.2 sulfhydryl groups/24,000 daltons and a value of 0.98 was obtained with ^{14}C-iodoacetamide. Thus it seems likely that each molecule of A contains only 1 half-cystine residue, and is therefore linked to Fragment B in nicked toxin by a single disulfide bridge. We have confirmed that intact toxin contains two disulfide bridges using the fluorimetric technique of Karush *et al.* (9). Therefore Fragment B must contain one internal disulfide bridge.

A very striking feature of Fragment A is its stability to heating and to extremes of pH. The fragment remains in solution and its activity is unchanged after incubation for 30 min either at 100° in buffer of neutral pH, or at 25° in buffers of pH 2 through 12 (10). In addition we have found that the activity of reduced Fragment A is not altered after reaction with iodoacetamide or chloromercuribenzoate, which indicates that the integrity of the free sulfhydryl group is not required for its activity.

In contrast to Fragment A, Fragment B exhibits a marked instability which renders it difficult to isolate and study. Fragment B rapidly precipitates out of solution at temperatures above 25°, and we have not succeeded in resolubilizing it except in protein denaturants such as sodium dodecyl sulfate or concentrated solutions of guanidine.

DISCUSSION

The purified preparations of diphtheria toxin that we have tested are composed predominantly of mixtures in various proportions of two species of protein, each of molecular weight about 63,000. One consists of single, intact polypeptide chains (intact toxin) and the other (nicked toxin) of two fragments of 24,000 and 39,000 daltons (A and B) linked by a disulfide bridge. We have shown that the former is converted into a

species similar to or identical with the latter when toxin is incubated with low concentrations of trypsin. This implies that the nicked toxin in our preparations is a derivative of the intact toxin, resulting from cleavage of the 63,000-dalton chains at a specific site presumably by proteases of the bacterial culture from which toxin was purified. This, in turn, indicates that the toxin is probably synthesized as a single 63,000-dalton polypeptide chain.

Although partial cleavage of intact toxin within cells of *Corynebacterium diphtheriae* cannot be ruled out, it seems likely that the toxin is released in the form of intact 63,000-dalton chains, and that nicking occurs through the action of a protease or proteases in the extracellular medium. We have found that the proportion of nicked chains in some of our highly purified preparations of toxin increases during storage over a period of weeks at 4°. This suggests that traces of proteases are present, presumably originating in the bacterial culture. Thus, the ratio of nicked to intact chains in any given preparation of toxin is probably a function of several variables, including the culture conditions of *C. diphtheriae*, the methods of purification of toxin, and the conditions and duration of storage, since proteolytic attack may occur to a variable extent during each of these stages.

We observed initially that a preparation of toxin containing approximately 80% intact 63,000-dalton chains exhibited a 4- to 5-fold increase in specific activity in catalyzing the ADP ribosylation of transferase II when treated either with trypsin or higher concentrations of two other less specific proteases, Pronase and subtilisin. Trypsin has been shown to effect similar increases in other preparations containing intact toxin chains, but preparations originally containing 100% nicked chains showed no change in specific activity.

We have determined the range of trypsin concentrations (0.2 to 1.0 μg per ml) optimal for enhancement of the specific activity and have analyzed the products of trypsin action. The enzymic activity of trypsin-treated toxin exhibits the same thiol-dependence as untreated toxin; both are virtually devoid of activity in the absence of thiol. After treatment with thiols almost 100% of the protein of trypsin-treated toxin dissociates into fragments which migrate on SDS gels identically with Fragments A and B from the nicked fraction of control toxin. The activity of trypsin-treated toxin after exposure to thiols appears to be due to Fragment A. This fragment from trypsin-treated or control toxin shows the same specific activity within experimental error (355 $\pm$ 15 enzyme units per μg), while Fragment B appears to lack enzymic activity.

These results support the conclusion that trypsin preferentially attacks the intact toxin chains at a specific site, converting them into species which are identical with the nicked fraction of untreated toxin. As indicated above, since Fragment A contains a single half-cystine residue, the fragment must be linked to Fragment B by a single disulfide bridge in the cleaved toxin chain. Since the entire toxin molecule contains two disulfide bridges, one of these must be located internally in Fragment B.

Our results are consistent with the idea that the preferential site of attack by trypsin in intact toxin is a single specific lysine or arginine residue. However, it is possible that attack at any of multiple basic residues within a narrow region may release fragments similar to A or B which could not be distinguished from one another by the methods we have employed. The fact that α-chymotrypsin does not activate intact toxin chains, while the relatively nonspecific proteases, Pronase and subtilisin, do activate the chains, is consistent with the idea that cleavage adjacent to a specific basic residue may be necessary for activation. We have not yet analyzed the products of cleavage by Pronase or subtilisin. The fact that the extent of activation of intact toxin declined at concentrations of trypsin above the optimal range of 0.2 to 1.0 μg per ml, while free Fragment A was unaffected at these concentrations suggests that trypsin-sensitive bonds which are obscured in free Fragment A are relatively exposed in whole toxin.

It is interesting that Fragment A is apparently catalytically active only after it has been dissociated from Fragment B, since there is evidence that the binding sites for both NAD^+ and transferase II are exposed on the whole toxin molecule. Everse *et al.* (11) have detected a ternary complex of molecular weight 114,000 containing stoichiometrically equivalent amounts of toxin, transferase II, and NAD^+. In addition, binding of NAD^+ to whole toxin has been reported, although it cannot be certain that the toxin preparations used were free from Fragment A (12). Thus, both transferase II and NAD^+ can bind to whole toxin, presumably to sites on the molecule corresponding to Fragment A, but the two substrates must not be positioned such that transfer of the ADP-ribose moiety of NAD^+ occurs at a measurable rate. After cleavage of the disulfide bridge linking Fragments A and B and dissociation, a conformational change must occur in Fragment A which renders it catalytically active. The sulfhydryl group of Fragment A is apparently not important in catalysis since blocking this group with thiol reagents does not affect the activity. It should be noted that we have not ruled out the possibility that the trace of activity of the whole toxin may be due to either intact or nicked toxin, and not contaminating Fragment A as we have suggested.

It is clear that Fragment A is not toxic, since it neither inhibits protein synthesis in intact HeLa cells nor produces symptoms of toxemia in guinea pigs. However, there is strong evidence that the reaction catalyzed by Fragment A occurs within cells treated with toxin, and it is probable that the resulting inhibition of protein synthesis is responsible for the toxicity of toxin. Gill *et al.* (2) have shown that when HeLa cells are treated for $\frac{1}{2}$ hour or more with toxin, extracts derived from these cells are markedly inhibited in their capacity to catalyze transfer of amino acids from aminoacyl-tRNA into protein. Furthermore this activity could be largely restored by treatment of the extracts with high concentrations of nicotinamide in the presence of added toxin, which presumably removed the ADP-ribosyl group from transferase II by reversal of the ADP ribosylation reaction. Moehring and Moehring (13) have made similar observations.

The most likely explanation for the difference in capacity of toxin and Fragment A to inhibit protein synthesis is that Fragment A cannot gain entry into the cytoplasm, whereas toxin can. This immediately suggests a possible role for Fragment B, or that portion of intact toxin corresponding to Fragment B. It would seem plausible that Fragment B might act specifically to bind whole toxin molecules to specific sites on the cell membrane, or otherwise to facilitate transport of the toxin into the interior of the cell. Binding of toxin to a limited number of binding sites on HeLa cells has been demonstrated (14), but it is not known what fraction of the bound toxin enters the cell, or how the toxin might effect entry. Regardless of the mechanism of entry it seems reasonable that the toxin must at

least penetrate the cell membrane in order to inactivate transferase II.

Once within the cell it is conceivable that whole toxin molecules might directly inactivate transferase II, perhaps as a result of activation through conformational changes which did not occur in our system *in vitro*. If the trace of activity which we have found in toxin is indeed due to whole toxin, either intact or nicked, then this activity might be sufficient to inhibit protein synthesis. However it seems likely that alterations in whole toxin may have to occur, corresponding to those reported in this and the preceding paper, before the enzymic activity can be expressed within cells.

Our results indicate that intact toxin chains must be first nicked at a specific site and subsequently dissociated into Fragments A and B in order for the enzymic activity to be fully expressed *in vitro*. It is quite possible that both these processes can occur within the body of a sensitive animal. Indeed, if our assumption is correct that intact chains are enzymically inactive *in vivo* as well as *in vitro*, then proteolytic attack on these chains must occur within the body, since we have found that the extent to which toxin is nicked apparently does not significantly affect its toxicity. Our results would be consistent with the proteolytic attack occurring either extracellularly or intracellularly. A cathepsin with the same specificity as trypsin has been reported (15).

The nicked toxin may be reduced within cells through the action of GSH or other reducing agents, resulting in dissociation, and hence release of active Fragment A. GSH is present in various animal tissues at concentrations ranging from approximately 1 to 6 mM (16), which should be sufficient to dissociate a high percentage of nicked toxin within an hour or less. Reduction of the nicked toxin extracellularly would be expected to lead to loss of toxicity, since Fragment A and presumably Fragment B are each not toxic. Our measurements of the toxicity of reduced, trypsin-treated toxin are in general agreement with this expectation, although a significant level of toxicity was retained by toxin treated in this manner. This may have resulted from incomplete reduction or incomplete dissociation of the reduced fragments. Reduction of toxin which had not been exposed to trypsin caused only a small decline in toxicity, apparently due to dissociation of the nicked fraction, which constituted 20% of the protein of the particular toxin tested. This indicates that intact toxin is resistant to inactivation by thiols, and supports the conclusion that intact toxin is indeed toxic.

Note Added in Proof—Results similar to those reported here and in the accompanying paper (3) have been obtained by workers at Harvard University. These are reported in two articles by D. M. Gill and L. L. Dinius (17) and by D. M. Gill and A. M. Pappenheimer, Jr. (18) in this issue of the Journal. We appreciate the cooperation of these authors in publishing their work simultaneously and in using the same nomenclature for the two forms of toxin and fragments thereof.

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REFERENCES

1. Honjo, T., Nishizuka, Y., Hayaishi, O., and Kato, I., *J. Biol. Chem.*, **243**, 3553 (1968).
2. Gill, D. M., Pappenheimer, A. M., Jr., Brown, R., and Kurnick, J. T., *J. Exp. Med.*, **129**, 1 (1969).
3. Collier, R. J., and Kandel, J., *J. Biol. Chem.*, **246**, 1496 (1971).
4. Drazin, R., and Collier, R. J., *Bacteriol. Proc.*, 88 (1970).
5. Smithies, O., *Science*, **150**, 1595 (1965).
6. Moore, S., *J. Biol. Chem.*, **238**, 235 (1963).
7. Erwin, V. G., and Pederson, P. L., *Anal. Biochem.*, **25**, 477 (1968).
8. Haber, E., and Anfinsen, C. B., *J. Biol. Chem.*, **236**, 422 (1961).
9. Karush, F., Klinman, N. R., and Marks, R., *Anal. Biochem.*, **9**, 100 (1964).
10. Kloppestech, K., Steinbeck, R., and Klink, F., *Hoppe-Seyler's Z. Physiol. Chem.*, **350**, 1377 (1969).
11. Everse, J., Gardner, D. A., Kaplan, N. O., Galasinski, W., and Moldave, K., *J. Biol. Chem.*, **245**, 899 (1970).
12. Goor, R. S., and Pappenheimer, A. M., Jr., *J. Exp. Med.*, **126**, 913 (1967).
13. Moehring, T. J., and Moehring, J. M., *J. Bacteriol.*, **96**, 61 (1968).
14. Pappenheimer, A. M., Jr., and Brown, R., *J. Exp. Med.*, **127**, 1073 (1968).
15. Fruton, J. S., Irving, G. W., Jr., and Bergmann, M., *J. Biol. Chem.*, **141**, 763 (1941).
16. Isherwood, F. A., *Biochem. Soc. Symp.*, **17**, 3 (1959).
17. Gill, D. M., and Dinius, L. L., *J. Biol. Chem.*, **246**, 1485 (1971).
18. Gill, D. M., and Pappenheimer, A. M., Jr., *J. Biol. Chem.*, **246**, 1492 (1971).