Structure and Activity of Diphtheria Toxin

I. THIOL-DEPENDENT DISSOCIATION OF A FRACTION OF TOXIN INTO ENZYMICALLY ACTIVE AND INACTIVE FRAGMENTS

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SUMMARY

The activity of diphtheria toxin in catalyzing transfer of the adenosine diphosphate ribose moiety of NAD\(^+\) into covalent linkage with the mammalian peptidyl transfer RNA translational factor, transerase II, is dependent upon exposure of the toxin to thiols. The toxin is almost completely inactive when assayed in the absence of thiols, but may be maximally activated by treatment with 50 mM dithiothreitol for 10 min.

The activation process has been correlated with certain structural features of the toxin. Studies involving electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate have led us to conclude that toxin consists of a mixture of two similar proteins of molecular weight about 63,000. One consists of intact, 63,000-dalton polypeptide chains (intact toxin), while the other (nicked toxin) consists of two fragments of 24,000 and 39,000 daltons (A and B, respectively) linked by at least one disulfide bridge. Treatment of toxin with thiols results in dissociation of the latter into Fragments A and B. Fragment A is enzymically active, and probably accounts for all the activity of thiol-treated toxin. Fragment B is almost certainly devoid of activity for reasons which are discussed, although this has not been demonstrated experimentally. Intact toxin appears to be inactive before or after treatment with dithiothreitol.

In the accompanying paper we show that intact toxin is a precursor of nicked toxin, and may be converted into the latter by treatment with trypsin.

Diphtheria toxin has been purified and characterized in a number of laboratories (1-4). It has been reported to be a protein of molecular weight 60,000 to 70,000 with a sedimentation constant of 4.2 to 4.6 S (5-8). The amino acid composition and end groups as well as other properties have been reported for crystalline preparations of toxin (9). However, no detailed analysis of the structure of the toxin molecule has appeared. This report is the first in a series from this laboratory which will be devoted to investigating the structure of diphtheria toxin in relation to its activities in vitro and in vivo.

The toxicity of diphtheria toxin is believed to result from its capacity to block protein synthesis. When a saturating concentration (~1 \(\mu\)g per ml) of the toxin is added to cultures of cells from sensitive animals, protein synthesis ceases at about 1/2 to 4 hours after the addition, depending on the strain of cells and culture conditions employed (10, 11). In cell-free systems from mammalian tissues, the toxin inhibits incorporation of amino acids into protein immediately provided the cofactor NAD\(^+\) is present (12). This inhibition has been shown to result from inactivation of transerase II, a soluble protein required for the GTP-dependent translocation of peptidyl transfer RNA from the "acceptor" to the "donor" site on ribosomes (13, 14).

Honjo et al. (15) and Gill et al. (16) have shown that in the presence of diphtheria toxin the adenosine diphosphate ribose moiety of NAD\(^+\) is transferred into covalent linkage with transerase II, producing an inactive derivative of the factor. The toxin acts catalytically in this reaction, in which nicotinamide and a proton are released concomitantly.

NAD\(^+\) + transerase II \(\rightarrow\) ADP-ribose-transerase II

\(\rightarrow\) nicotinamide + H\(^+\)

Studies by Moehring and Moehring (17) and Gill et al. (16) strongly support the suggestion that this reaction is responsible for the inhibition of protein synthesis by toxin in intact cells.

Here we report that the toxin is virtually without enzymic activity in this reaction unless it has been exposed to thiols. The toxin can be maximally activated by incubation with 50 mM DTT\(^-\) for 10 min and remains active when diluted and assayed in very low thiol concentrations.

The activation by thiols is shown to be related to dissociation of a fraction of the toxin protein into fragments. Electrophoresis of toxin on polyacrylamide gels in the presence of sodium dodecyl sulfate has shown that most preparations consist of mixtures of two similar proteins, each of molecular weight 62,000 to 63,000. One of these (intact toxin) apparently consists of intact, 63,000-dalton chains which are enzymically inactive both before and after exposure to thiols. The other (nicked toxin) consists of two fragments of 24,000 and 39,000 daltons (A and B, respectively) linked by at least one disulfide bridge. Treatment of toxin with thiols results in dissociation of the latter into Fragments A and B. Fragment A is enzymically active, and probably accounts for all the activity of thiol-treated toxin. Fragment B is almost certainly devoid of activity for reasons which are discussed, although this has not been demonstrated experimentally. Intact toxin appears to be inactive before or after treatment with dithiothreitol.

In the accompanying paper we show that intact toxin is a precursor of nicked toxin, and may be converted into the latter by treatment with trypsin.

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

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3 Recipient of Graduate Fellowship GM 41952 from the National Institutes of Health.
two fragments, A and B, of molecular weight 24,000 and 39,000, respectively, linked by at least one disulfide bridge. When toxin is treated with thiols, the nicked toxin dissociates into Fragments A and B. Fragment A is enzymically active, and is probably responsible for all the activity of thiol-treated toxin. Fragment B is believed to be inactive for reasons which are stated in the “Discussion.”

In the accompanying paper we show that intact and nicked toxin are in fact related proteins. Our results imply that the latter arises by proteolytic attack at a specific site within intact toxin chains. In referring to the products of dissociation by thiols the term fragment has therefore been used in preference to the term subunit, which was employed in preliminary reports of part of this work (18, 19). Fragment A is identical with the 2.5 S “subunit” described in those reports.

Experimental Procedures

Reagents—Dithiothreitol was purchased from Calbiochem, Sephadex from Pharmacia, DEAE-cellulose from Mann, and acrylamide gel reagents from Bio-Rad (Richmond, California). 3H-ATP was obtained from New England Nuclear. All other chemicals were purchased from commercial sources and were of the highest grade generally available.

Diphtheria Toxin—Most of the experiments in this and the following paper were performed with a single preparation of diphtheria toxin (lot 007) produced by growth of Corynebacterium diphtheriae (Park Williams strain 8) in still culture in Roux bottles (20). After removal of the bacteria by centrifugation of the culture for 10 min at 23,000 × g, the toxin was harvested from the supernatant by collecting that fraction which precipitates between 40 and 60% saturated (NH4)2SO4. This and all subsequent operations were performed at 0-4°C. The precipitate (3.3 g of protein) was dissolved in 118 ml of 5 mM sodium phosphate buffer, pH 6.8, and dialyzed for 4 days against four changes of the same buffer. The dialysate was applied to a column of DEAE-cellulose (1.8 × 31 cm) equilibrated with this buffer, and after washing with 250 ml of the buffer, the toxin was eluted with a linear gradient of phosphate buffer, between 0.005 and 0.2 M. A single asymmetrical peak of protein emerged and extended tailing of the peak was observed. The ADP ribosylation activity was approximately proportional to the protein concentrations of the fractions throughout this region. The active fractions were pooled, concentrated by dialysis against 3 volumes of saturated ammonium sulfate and further purified by chromatography on Sephadex G-100 as described under Fig. 1. The peak labeled Tz was found to contain protein with a sedimentation coefficient of about 4 S, characteristic of toxin. Fractions between 420 and 585 ml were pooled, and after lyophilization, the material was rechromatographed on a column of Sephadex G-100, (superfine) (2.5 × 87 cm). The protein in the central area of the major peak from this column, which was constant in specific activity within ±10%, was pooled and stored at -70°C. The final product (360 mg) had a specific toxicity of approximately 20 guinea pig minimum lethal doses per pg of protein.

Fragment A—Fragment A was derived from purified toxin which had been incubated in 50 mM Tris-HCl-1 mM EDTA for 1 hour in the presence of 0.1 M dithiothreitol followed by repeated chromatography on superfine Sephadex G-100. The final product was pure as judged by electrophoresis on SDS acrylamide gels.

Radioactive NAD+—NAD+ uniformly labeled with 3H in the adenosine moiety was synthesized from NMN and 3H-ATP using NAD-ribosylationase prepared from hog liver by the method of Kornberg (21). The reaction mixture (1.07 ml) contained 90 μmoles of Tris-HCl buffer, pH 7.5 (30°C), 15 μmoles of MgCl2, 200 μmoles of nicotinamide, 5 μmoles of NMN, 120 μmoles of 3H-ATP (419 μCi per pmole), 3.7 μmoles of phosphoenolpyruvate, 10 μg of pyruvate kinase, and 200 μl of freshly prepared enzyme. After incubation for 1 hour at 37°C, the mixture was heated for 90 sec in a boiling water bath, and the resulting precipitate was removed by centrifugation. The precipitate was resuspended in 1 ml of water and recentrifuged, and the combined supernatants from the two centrifugations were applied to a column (80 × 1 cm) of DEAE-cellulose equilibrated with 2 mM sodium phosphate buffer, pH 6.0. After washing the column with 100 ml of the same buffer, the NAD+ was eluted with 5 mM phosphosphate buffer, pH 6.0. The NAD+ emerged from the column as a symmetrical peak (approximately 45 ml). The measured specific radioactivity was the same as that of the ATP used. The preparation was diluted to 2.5 μM and was stored at -70°C in 1-ml portions.

Transerase II—A partially purified preparation of transerase II was employed which was obtained by collecting that fraction of ribosome-free supernatant from rabbit reticulocytes which precipitates between 40 and 60% saturated ammonium sulfate (Fraction AS 40-60) (13). The final product was dialyzed against buffer containing 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 1.0 mM DTT in the cold and stored at -70°C in 1-ml portions. For assays conducted in the absence of thiols, DTT was removed from Fraction AS 40-60 immediately before...
the experiment by passing it through a small column of Sephadex G-25 equilibrated with the same buffer lacking DTT.

The concentration of transferase II was estimated by incubating various amounts of Fraction AS 40-60 in reaction mixtures identical with those used for assay of ADP ribosylation activity, either for 100 min in the presence of 50 enzyme units of toxin or 20 min in 3000 enzyme units of toxin. An enzyme unit of toxin is defined in the first paragraph of the "Results." Transferase II (up to at least 5 pmol) was reacted completely under these conditions, and the quantity of the factor was calculated from the incorporated radioactivity assuming that each molecule of the factor is capable of accepting one ADP-ribose group.

**Assay of ADP Ribosylation Activity**—Samples of toxin were assayed in reaction mixtures (250 μL) containing 12.5 pmoles of Tris-HCl buffer, pH 8.2, 25 nmoles of EDTA, 10 μmoles of dithiothreitol, 12.5 pmoles of transferase II, 12.5 pmoles of 14C-NAD+, and 1 to 5 enzyme units of toxin. All the components except NAD+ were mixed at 0°C, and after initiation of the reaction by addition of the NAD+, the mixtures were incubated at 25°C for 15 min. The reaction was terminated by addition of 250 μL of 10% trichloroacetic acid, and the precipitate was collected by filtration on a 2.4-cm Whatman GF/C glass fiber disc, and washed with four 5-ml portions of 5% trichloroacetic acid. The discs were finally attached to aluminum planchets with melt, and counted in a Nuclear-Chicago low background counter.

Identical samples within the same experiment generally fell within ±5% of the mean. Greater deviations, up to about +20%, were observed in separate experiments. These deviations were in some instances due to variations in different preparations of transferase II.

**SDS Polyacrylamide Gel Electrophoresis**—Polyacrylamide gels containing 10% acrylamide and 0.27% methylenebisacrylamide, in sodium phosphate buffer, pH 7.0, 1% in sodium dodecyl sulfate, were prepared according to the method of Weber and Osborn (22). Each gel (0.6 cm), a sample of about 10 μg of protein in 50 to 100 μL of 0.01 M sodium phosphate buffer, pH 7.0, 1% in sodium dodecyl-sulfate, was heated for 1 min at 100°C. If reduction was desired, β-mercaptoethanol was added to 5% prior to heating. Finally, 1 drop of glycerol was added and the samples were layered under the buffer directly on the gels. Electrophoresis was performed at room temperature for 4 hours at a constant current of 10 ma per gel, using the buffer system described by Weber and Osborn (22). The gels were soaked for 15 min in 10% trichloroacetic acid, stained for at least 5 hours in Coomassie brilliant blue (22), and destained by diffusion in a solution of 10% methanol and 10% acetic acid.

Protein contents of the bands were estimated by scanning the stained gels at 550 nm in a Gilford spectrophotometer equipped with a linear transport device.

**Molecular Weight Determination**—The molecular weights of the unknowns were determined by comparison with this plot. The values of the molecular weight shown in the text are the mean of three determinations. The error shown is the average deviation.

Protein was estimated either by the method of Lowry et al. (23) or calculated from the absorbance at 260 and 280 nm (24).

**RESULTS**

**Characteristics of ADP Ribosylation Reaction**—The activity of diphtheria toxin in catalyzing the ADP ribosylation of transferase II was assayed by measuring incorporation of label from 14C-NAD+, uniformly labeled in the adenosine moiety, into acid-insoluble material in the presence of partially purified transferase II from rabbit reticulocytes. The reaction mixtures contained Tris-HCl buffer, pH 8.2 (50 mM), EDTA (0.1 mM), and dithiothreitol (40 mM), in addition to transferase II (50 nm), 14C-NAD+ (50 nm), and toxin (0 to 6 nm). The reaction was conducted at 25°C, which is the approximate center of a 10°C region of temperature optimum. We have confirmed the report that the optimal pH is 8.2 (25). An arbitrary enzyme unit of toxin has been defined as the amount which catalyzes incorporation of 1 pmole of ADP-ribose into acid-insoluble material in 15 min under these conditions.

The reaction was almost linearly dependent on the concentration of toxin up to 3 enzyme units per ml. The concentrations of NAD+ and transferase II employed were well below saturating levels. NaCl, KCl, and NH4Cl, all inhibited the reaction by 25 to 40% at a concentration of 30 mM, and 65 to 80% at 100 mM. MgCl₂ and magnesium acetate were each approximately 10-fold more effective as inhibitors on a molar basis. We have not confirmed the slight stimulation of the reaction by MgCl₂ at 0.5 mM which has been reported (26). The reaction has been found to be inhibited by a variety of species of RNA, and the inhibition by RNA is partially relieved by MgCl₂ at concentrations as low as 0.5 mM. The reported stimulation by MgCl₂ may therefore have resulted from the presence of RNA in the preparation of transferase II employed.

The experiments below, except where specified, were performed with a single preparation of toxin (lot 007) prepared as described under "Experimental Procedures." This preparation is compared with others toward the end of the "Results."

**Dependence of ADP Ribosylation Reaction on Thiols**—As shown in Fig. 2 the ADP ribosylation reaction catalyzed by toxin is strongly dependent on the concentration of thiol present in the assay mixture. The rate of reaction increases markedly as the concentration of DTT is increased, up to about 20 mM. The reaction is maximal and constant between 30 and 50 mM, and declines at higher concentrations of the thiol. The concentration of DTT routinely employed in our assays was 40 mM.

In order to obtain a more accurate figure for the activity in the absence of thiol, a titration with DTT in the range of 0 to 2.0 mM was conducted in the presence of a 10-fold higher concentration of toxin (inset, Fig. 2). The activity per unit of toxin in the absence of thiol was calculated to be approximately 0.004 times that at 20 mM DTT, and no discontinuity was evident in the thiol-dependence curve.

**Kinetics of ADP Ribosylation Reaction**—The kinetics of the ADP ribosylation reaction are consistent with the activation of one or more components of the reaction mixture by thiols. As

* J. A. Traugh and R. J. Collier, unpublished results.
shown in Fig. 3, a kinetic lag is observed which diminishes as the concentration of thiol is increased. At the concentration of 40 mM DTT routinely used in our assay mixtures, the reaction followed linear kinetics after a short lag of 1 to 1½ min. At higher concentrations of thiol, the lag was reduced further, but the rate of reaction following the lag was lower than that at 40 mM DTT. It may be that the inhibition of the reaction at high thiol concentrations is due to the increase in ionic strength, since the ionization of thiols is significant at the pH employed.

Activation of Toxin by Thiols—Prior incubation of either transferase II or 14C-NADf with moderate concentrations of DTT (5 mM) caused no increase in the rate of the reaction when the assay was conducted in low concentrations of thiol. In contrast, incubation of toxin under the same conditions produced a marked increase. For example, when a sample of toxin was treated with 50 mM DTT for 30 min at 25° and subsequently diluted and assayed in the presence of 5 µM DTT, the reaction proceeded linearly at a rate similar to that of untreated toxin assayed in the presence of 40 mM DTT (Fig. 3). The activity of untreated toxin in 40 µM DTT was virtually nil (Fig. 2, inset).

Kinetic curves of the activation of the toxin in the presence of various concentrations of DTT are shown in Fig. 4. Concentrated samples of toxin were treated at 25° with DTT at the concentrations indicated, and were diluted at the times shown by a factor of 1000 in thiol-free buffer and assayed in the presence of 40 µM DTT. The activation was complete by 10 min in the presence of 50 mM DTT or 60 min in 10 mM DTT. The initial rate of activation was approximately proportional to the concentration of thiol.

Cysteine, β-mercaptoethanol, and GSH were also shown to activate the toxin, but DTT was more than twice as effective as any of these on a molar basis. The oxidized (disulfide) forms of these compounds were without effect.

Electrophoresis of Toxin in SDS Gels—It has been found that proteins electrophoresed in polyacrylamide gels in the presence of sodium dodecyl sulfate migrate as an inverse function of molecular weight (27). The toxin, after treatment with 1% sodium dodecyl sulfate at 100° for 1 min, migrated as a single band (Band T, Fig. 5). A molecular weight of 62,300 ± 1,700 was estimated from the mobility, by comparison with a plot of log molecular weight versus mobility for a series of standard proteins of known molecular weight (22). When 5% β-mercaptoethanol was added to toxin before it was heated in sodium dodecyl sulfate, the intensity of Band T decreased slightly, and...
two new bands appeared at positions corresponding to molecular weights of approximately 24,200 ± 500 (Band A) and 39,200 ± 800 (Band B). The sum of the molecular weights of A and B is 63,400 which is approximately the same as that of the toxin band. Thus we assume that these bands arose by dissociation of protein molecules containing one A and one B fragment linked by one or more disulfide bridges. Below we refer to whole toxin as having a molecular weight of 63,000, which is approximately the sum of Fragments A and B and is well within the experimental error of our determinations for whole toxin. Approximately 80% of the protein in the preparation did not dissociate under these conditions, and the percentage was not changed by increasing either the concentration of mercaptoethanol or the duration of heating of the samples prior to electrophoresis. This material is assumed to consist of intact 63,000-dalton polypeptide chains.

**Fractionation of Thiol-treated Toxin by Gel Filtration**—When toxin was chromatographed on a column of Sephadex G-100 (superfine mesh), it emerged as a single peak of protein with the ADP-ribosylation activity coincident (Fig. 6A). Toxin which had been treated with 100 mM DTT at 25° for 1 hour (Fig 6B) exhibited a profile of protein with differences which could be correlated with the pattern of bands of thiol-treated toxin on SDS gels shown in Fig 5. Thus most of the protein emerged at the same position as untreated toxin (34 ml), and this material was shown on SDS gels to contain mostly intact 63,000-dalton chains, plus a small amount of nicked toxin which dissociated into Fragments A and B when treated with mercaptoethanol. The small peak of protein at 43 to 44 ml contained mostly Fragment A plus small amounts of Fragment B. The region in between these two peaks contained Fragment B plus tailings from the protein peaks. The recovery of protein in the column fractions was 98% with untreated toxin and 93% with thiol-treated toxin. The difference appeared to be due to loss of Fragment B in thiol-treated toxin, which is consistent with the instability of this fragment we have observed (28).

The pattern of activity in the column fractions from thiol-treated toxin showed interesting differences from the control. Approximately 84% of the total activity was associated with the protein peak of Fragment A, and only 11% remained associated with 63,000-dalton material (Peak T). This suggested that the activity of thiol-treated toxin might be due entirely to Fragment A. We have subsequently purified Fragment A and found that it is highly active in the absence of B. Assuming that Fragment A is the only active species, the activity of Peak T can be attributed to Fragment A released from the dissociable toxin remaining. The activity in the regions between Peaks T and A can be accounted for by Fragment A in the form of both...
dimer of A and 63,000-dalton nicked toxin, both of which were detected on SDS gels run in the absence of thiols. These apparently formed through association of the free fragments and autoxidation during passage through the column.

Our results do not exclude the possibility that Fragment B is enzymically active, but this is considered highly unlikely for reasons stated in the "Discussion."

Thiol-dependence of Activity of Fragment A—Fragment A, in contrast to untreated toxin, is partially active in the absence of thiols (Fig. 6B). The purified fragment exhibits maximal activity in the range of 0.5 to 5 mM DTT, and in the absence of thiols the activity is approximately 50% of maximal. A gradual decline in activity is observed as the thiol concentration is raised above 5 mM; the decline continues at approximately the same slope through 100 mM DTT. For comparison, the dashed line in Fig. 7 represents the activity of toxin (lot 007) as a function of the concentration of DTT.

Considerable variation was observed from experiment to experiment in the ratio of the activity of Fragment A in the absence of thiols to that at moderate concentrations of DTT (40 mM DTT) (see Peats A, Figs. 1 and 6B). The ratio, which ranged from approximately 0.4 to 0.9, may have varied according to the degree of dimerization of the fragment. In addition, the activity of the fragment in some experiments near the maximal only at concentrations of DTT as high as 2 to 3 mM, perhaps for the same reason.

Kinetics of the reaction catalyzed by Fragment A were linear in the presence of 2 mM or 40 mM DTT, and showed no detectable lag. In the absence of thiols the rate of the reaction was similar initially to that in 2 mM DTT, but declined markedly over the 15-min period of the experiment. This suggests that thiols may serve to protect either transferase II or Fragment A, or both, from inactivation through oxidation.

Other Preparations of Toxin—Preparations of toxin from other laboratories as well as preparations other than lot 007 from our own laboratory have been analyzed on SDS gels and found to give band patterns qualitatively similar to lot 007. In the absence of thiol each of these showed a major 63,000-dalton band and only small amounts of other bands, if any. In every case except one (Gel 2, Fig. 8), a fraction of the 63,000-dalton protein was dissociated by thiols into fragments similar to or identical with A and B. The fraction of nicked toxin ranged from 0 to virtually 100%. In all the preparations of toxin produced in our laboratory the percentage of nicked toxin was close to 20%. In every case the activity in the absence of thiol was less than 5% of that in 40 mM DTT.

DISCUSSION

The activity of diphtheria toxin in catalyzing the ADP ribosylation of transferase II is strongly dependent on the presence of thiols. The activity of toxin Preparation 007 used here in the absence of thiols was less than 1/200 that at 40 mM DTT, at which the activity is maximal. If exposed to thiols prior to assay, the toxin retains activity when the assay is conducted in an extremely low concentration of thiol.

Fractionation of toxin by chromatography on Sephadex G-100 and electrophoresis on SDS gels has shown that treatment with thiols results in dissociation of a fraction of the 63,000-dalton whole toxin, into fragments of molecular weight 24,000 (A) and 39,000 (B). The remainder of the protein, which constitutes between 0 and almost 100% of the total in various preparations, remains in the form of intact 63,000-dalton chains as judged by migration on SDS gels. In the preparation (007) employed in most of these experiments, 20% of the protein could be dissociated with thiols.

Most of the activity of thiol-treated toxin was shown by Sephadex chromatography to be associated with the 24,000-dalton Fragment A, which is identical with the 2.5 S subunit we reported earlier in studies with sucrose gradients (18). We suggest that Fragment A may be entirely responsible for the activity of thiol-treated toxin. A small amount of activity
remained associated with the peak of 63,000-dalton protein, but this could be attributed to Fragment A released from a small amount of dissociable toxin which had apparently escaped reduction prior to chromatography. Additional low levels of activity in the intermediate molecular weight range were probably due to Fragment A in the form of dimer of A, and nicked toxin, both of which apparently formed by autoxidation of fragments which associated during chromatography. The results of our experiments on activation of toxin by thiols and the kinetics of the reaction in the presence of various concentrations of thiols are consistent with the idea that the activation of toxin depends on dissociation of the nicked fraction into Fragments A and B.

Fragment A has been purified and shown to be highly active in catalyzing the ADP ribosylation of transferase II in the absence of Fragment B. Free Fragment A was found to exhibit maximal activity in the presence of 0.2 to 5 mM DTT (and in some experiments even lower concentrations), as compared with 30 to 50 mM DTT required for intact toxin. A low concentration of DTT may be necessary to dissociate or prevent the formation of dimers of A, which could conceivably be lower in activity than free A, or to maintain sulfhydryl groups of transferase II in a reduced state. Thiols are required for activity of transferase II in protein synthesis and may be necessary for its maximal activity as a substrate in the ADP ribosylation reaction. The activity of Fragment A does not depend on the integrity of its sulfhydryl group, since its activity has been found to be unaffected by reaction with sulfhydryl reagents (28).

The range of concentrations of DTT in the assay mixture, in which toxin exhibits maximal ADP ribosylation activity appears to be determined by two antagonistic effects of the thiol, namely (a) activation of toxin by release of Fragment A, and (b) inhibition of the ADP ribosylation reaction catalyzed by the released fragment. As the concentration of thiol is raised the initial kinetic lag due to the time required for dissociation of nicked toxin is shortened, but the final rate of reaction is decreased. Thus the kinetic lag is decreased below 1 min at concentrations of DTT above 100 mM, but the extent of the reaction in 15 min declines due to the predominant inhibitory effect of the thiol on the reaction. Inhibition of the reaction by high concentrations of DTT, which was observed with purified Fragment A as well as toxin, may result from the increase in ionic strength due to ionization of the sulfhydryl groups at the pH employed (8.2) since the reaction appears to be sensitive to increases in ionic strength.

Although most if not all of the activity can be accounted for by Fragment A in the various column fractions, we cannot exclude the possibility that Fragment B may also exhibit a low level of activity. Due to the instability of this fragment, as reported in the following paper, we have been unable to isolate it in native form in order to test its activity. However, we feel that this fragment is almost certainly devoid of activity for two reasons. (a) Fragments A and B have distinctly different properties (28); (b) it is now known that Fragments A and B are derived from complementary and almost certainly nonoverlapping regions of single 63,000-dalton polypeptide chains. In the following paper we show that the intact 63,000-dalton chains in the preparations of toxin tested are precursors of the dissociable fraction, and may be converted into the latter by treatment with trypsin. In order for Fragment B to exhibit catalytic activity similar to Fragment A, the potential binding sites for both transferase II and NAD+ would presumably have to be present in duplicate within the 63,000-dalton toxin chain. This seems highly unlikely.

We have not excluded the possibility that the low level of activity of untreated toxin (less than 1/200 that of thiol-treated toxin) may be due to either the intact or nicked 63,000-dalton molecules. However, it seems likely that trace amounts of free Fragment A in the preparation may account for the activity. Relatively large amounts of Fragment A, in terms of activity, are found in partially purified toxin (Fig. 1), and our methods of purification may not have been sufficient to completely free the toxin from this contaminant. The fragment was not detected on SDS gels, and probably could not have been, since we have calculated that 4 ng of Fragment A per 10 pg of toxin could have accounted for the observed activity. The free fragment may have arisen in culture through disulfide interchange of nicked toxin with cystine in the medium, catalyzed by traces of thiols released from cells.

Other preparations of toxin which were tested were similar to lot 007 in that most consisted primarily of mixtures of nicked and intact 63,000-dalton chains. One contained no intact fraction, while another consisted almost entirely of intact chains; others contained intermediate amounts. Fragments A and B were the major dissociation products in every case. Maximal activity of all the preparations was expressed only in the presence of thiol. In no case was the activity in the absence of thiol greater than 5% of maximal.

The effects of thiols on the toxicity of toxin are considered in the following paper (28). There we speculate on the possible significance of these results in relation to the events leading to inhibition of protein synthesis by toxin in sensitive animal cells.

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