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Expression of Enzymic Activity by Exotoxin A from *Pseudomonas aeruginosa*

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Exotoxin A from *Pseudomonas aeruginosa* is a single polypeptide chain (M_r, 66,000) containing little if any adenosine 5'-diphosphate ribosyltransferase or oxidized nicotinamide adenine dinucleotide glycohydrolase activity. These activities have been demonstrated in the reduced intact toxin and in a peptide (M_r, 26,000) isolated from culture fluids or toxin preparations after storage. In this report we describe methods for generating enzymically active fragments by cleaving the fully or partially reduced exotoxin by proteolytic or chemical methods. Incubation of reduced toxin with chymotrypsin in the presence of oxidized nicotinamide adenine dinucleotide yielded an enzymically active peptide (M_r, 26,000) similar to the fragment characterized previously. Chemical cleavage by treatment of the reduced molecule with CNBr or 2-nitro-5-thiocyanobenzoate yielded fragments (M_r, 50,000 and 30,000, respectively) with similar activities. Also both adenosine 5'-diphosphate ribosyltransferase and oxidized nicotinamide adenine dinucleotide glycohydrolase activities were maximally expressed by the intact exotoxin after reduction of only two of its four disulfide bridges. Kinetic constants for activated whole toxin were similar to those of fragment A of diphtheria toxin. It is evident that in the native toxin the catalytic center is buried or distorted and that alterations in the covalent structure permit the center to become exposed or assume an active configuration. It is unknown whether reduction, proteolytic processing, or both occur during the course of toxin action on whole cells.

Most clinical isolates of *Pseudomonas aeruginosa* produce a protein toxin, exotoxin A (M_r, 66,000), which may be a virulence factor during infections of humans or experimental animals (13). Like diphtheria toxin, exotoxin A inhibits protein synthesis by catalyzing transfer of the adenosine 5'-diphosphate (ADP) ribosyl moiety of oxidized nicotinamide adenine dinucleotide (NAD^+) into covalent linkage with elongation factor 2 (EF-2) (6). There is little doubt that the cytocidal and lethal effects of both of these toxins are due to this reaction.

The secreted forms of both diphtheria toxin and exotoxin A are proenzymes, which must undergo alterations in covalent structure before ADP-ribosyltransferase activity is expressed (2, 11, 12, 18). In diphtheria toxin (M_r, 60,000) this activity may be elicited by cleavage of the intact molecule by limited proteolysis and reduction into two major fragments, fragments A and B. Fragment A (amino terminal; M_r, 21,145) catalyzes both ADP-ribosylation of EF-2 and hydrolysis of NAD^+ (NAD^+-glycohydrolase; EC 3.2.2.5). However, it is virtually devoid of toxicity unless attached to the complementary B fragment. Fragment B (M_r, 39,000) has no known enzymic activity. However, it functions in attachment of the toxin to specific cell surface receptors and may serve other essential functions in promoting transfer of the catalytic center of the fragment A moiety across the plasma membrane (3).

In the case of exotoxin A, an enzymically active proteolytic fragment is known to be generated under certain conditions, but ADP-ribosyltransferase activity is also expressed by the intact molecule after reduction. The active fragment (M_r, 26,000), which exhibits both ADP-ribosyltransferase and NAD^+-glycohydrolase activities, has been isolated from culture fluids and is found in some preparations of purified toxin after storage (2, 18). Although it is virtually certain that this peptide is generated by the action of traces of bacterial protease(s) on the toxin, attempts to produce such a fragment with known proteases have been unsuccessful until now. Here we report a method for generating this or an almost identical active fragment by treating the fully or partially reduced toxin with chymotrypsin in the presence of NAD^+. We also describe procedures for preparing other active fragments by chemical cleavage.

Previous work has shown that exotoxin A becomes enzymically active after exposure to reducing agents in the presence of chaotropic agents, such as urea (12, 18). The fact that no proteolytic cleavage of the 66,000-dalton molecule has been detected under these conditions implies that the catalytic center can assume an active configuration and be exposed in the partially unfolded, intact molecule. We present data which demonstrate that both ADP-ribosyltransferase and NAD^+-glycohydrolase activities are maximally expressed after reduction of only about two of the four disulfide bridges within the toxin. The enzymic properties of the activated whole toxin and the 26,000-dalton chymotryptic peptide are similar to those of fragment A of diphtheria toxin.

MATERIALS AND METHODS

Production and purification of exotoxin A. *P. aeruginosa* strain PA 103 was grown in a 40-liter carboy in dialyzed Trypticase soy broth supplemented with 1% glycerol and 50 mM monosodium glutamate (14). The culture was maintained at 32°C under vigorous aeration with pure oxygen. At the onset of the stationary phase, the cells were removed by centrifugation at 10,000 × *g* for 45 min. The toxin was purified from the culture fluid by a modification of the procedure of Liu et al. (15). After successive precipitations with zinc acetate and ammonium sulfate, the crude preparation was dialyzed against 10 mM Tris-(hydroxymethyl)aminomethane (Tris)hydrochloride buffer (pH 8)-1 mM ethylenediaminetetraacetate (EDTA). The dialyzed material (250 ml) was then passed through a 40-ml hemoglobin-Sepharose 4B column to remove contaminating proteases (16) and chromatographed on a column of diethylaminoethyl cellulose equilibrated with 10 mM Tris-hydrochloride (pH 8)-1 mM EDTA. The toxin peak, which was identified by high levels of ADP-ribosyltransferase activity, was pooled and concentrated by precipitation with 60% ammonium sulfate. The precipitated toxin was dissolved in 10 mM Tris-hydrochloride buffer (pH 8)-1 mM EDTA and chromatographed on a column of Sephacryl S-200 (2.6 by 79 cm) equilibrated in 10 mM Tris-hydrochloride buffer-1 mM EDTA, pH 8 (flow rate, 22 ml/h). The toxin was concentrated by precipitation with 60% ammonium sulfate, dissolved in 10 mM sodium phosphate buffer (pH 7)-1 mM EDTA, and then applied to a column (2.6 by 8 cm) of diethylaminoethyl cellulose equilibrated in the same phosphate buffer (flow rate, 9.2 ml/h). Adsorbed material was eluted with a 0 to 0.25 M gradient of sodium phosphate. Exotoxin A prepared in this manner gave a single band on polyacrylamide gel electrophoresis in the presence or absence of sodium dodecyl sulfate (SDS).

Sulfhydryl determination. Exotoxin A (50-μl samples; 0.2 mg/ml) was incubated with varing concentrations of urea and 1 mM dithiothreitol (DTT) for 30 min under a steady stream of nitrogen. [^{3}H]iodoacetic acid was added in twofold molar excess over DTT and allowed to react for 15 min at room temperature in the dark. The reaction was stopped by adding 10 μl of 0.4 M cysteine hydrochloride, and the samples were brought to 100 μl with water; 10 μl of a solution of bovine serum albumin (2 mg/ml) was added, and 50-μl portions were processed for counting by precipitation of the protein in filter paper with trichloroacetic acid as described previously (1). Samples were counted by liquid scintillation. As a control, 0.4 M cysteine hydrochloride was added to the toxin solution before the addition of [^{3}H]iodoacetic acid. The radioactivity incorporated in the presence of cysteine was subtracted from the radioactivity of the experimental samples.

Measurements of the ADP-ribosyltransferase activity of samples of exotoxin A treated with urea were made on samples that had been incubated with nonradioactive iodoacetic acid under the conditions described above. Reaction with this reagent was stopped by 1,000-fold dilution with 50 mM Tris-hydrochloride buffer (pH 8.2) containing 0.2 mg of bovine serum albumin per ml.

Chemical cleavage of exotoxin A. Exotoxin A was treated with 2-nitro-5-thiocyanobenzoate (TNB-CN) in the presence of 6 M guanidinium hydrochloride-5 mM DTT, as described by Jacobson et al. (7). Cleavage with cyanogen bromide was accomplished by dissolving 3 mg of lyophilized exotoxin A in 1 ml of 70% formic acid, followed by the addition of 20 μmol of solid cyanogen bromide. After 12 h of incubation at room temperature, the sample was diluted by adding 2 ml of water and lyophilized.

Enzyme assays. The ADP-ribosyltransferase assay was performed with wheat germ EF-2, as described by Chung and Collier (1). For determinations of kinetic parameters, the incubation time was shortened to 7 min and the exotoxin A concentration was held between 5 and 10 nM. NAD^+-glycohydrolase activity was measured by ethyl acetate extraction of the [^{14}C]nicotinamide released by hydrolysis of nicotinamide [^{14}C]NAD^+ (8).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of SDS was performed as described by Laemmli (10) in the apparatus of Reid and Bieleski (17). We routinely denatured proteins by boiling them for 5 min in the presence of 0.1% SDS, although the relative mobilities of exotoxin A and the various fragments were unaffected by heating. For analyses of enzymic activity, proteins were incubated with 0.1% SDS at room temperature for 30 min.

Diphtheria toxin and fragment A. Partially purified diphtheria toxin was purchased from Connaught Laboratories, Toronto, Canada. Its further purification and the preparation of fragment A have been described previously (4).

Radiochemicals and other reagents. Nicotinamide [^{14}C]NAD^+ was obtained from Amersham/Searle Corp., Des Plains, Ill., and had a specific activity of 22 mCi/mmol. NAD^+ labeled in the adenine portion of the molecule was synthesized as described previously (9). [^{3}H]iodoacetic acid was purchased from New England Nuclear Corp., Boston, Mass. It was diluted with nonradioactive iodoacetic acid, and the specific activity was determined by alkylation of reduced ribonuclease.

Chymotrypsin was purchased from Worthington Biochemicals Corp., Freehold, N.J. TNB-CN was prepared as described by Degani and Patchornik (5). Cyanogen bromide, monosodium glutamate, and NAD^+ and its analogs were obtained from Sigma Chemical Co., St. Louis, Mo.; urea was from Schwarz/Mann, Orangeburg, N.Y., and DTT was from Vega-Fox Biochemicals, Tucson, Ariz. Trypticase soy broth was from BBL Microbiology Systems, Cockeysville, Md.

RESULTS

Active fragment generated by treatment with chymotrypsin. Chung and Collier described the purification and characterization of a 26,000-dalton polypeptide isolated from culture fluids of *P. aeruginosa* strain PA 103 (2). Because similar or identical peptides had been found in preparations of purified toxin after storage (18), the peptide was assumed to be a proteolytic fragment from exotoxin A. However, initial attempts to generate the peptide by treatment of the toxin with known proteases resulted in failure. Treatment of the native or reduced molecule with concentrations of trypsin, pancreatic elastase, or pronase sufficient to alter the molecular weight resulted in rapid degradation. Limited digestion of the native molecule with chymotrypsin (200 μg/ml) produced a few fragments having molecular weights of 45,000 or more, but none in the range of 26,000. Similar digestion of the reduced molecule caused rapid degradation and loss of activity. Carboxypeptidase A, carboxypeptidase B, or a combination of the two had no significant effect on the toxin.

A successful set of conditions was found when chymotryptic digestion was performed on reduced toxin in the presence of NAD^+. As Fig. 1 shows, the ADP-ribosyltransferase activity of exotoxin A which had been treated with 4 M urea and 1 mM DTT was preserved if NAD^+ was present in the chymotryptic digestion mixture, but was rapidly lost in the absence of NAD^+. The relative protective activities of the various NAD^+ analogs correlated with their respective inhibition constants for the ADP-ribosyltransferase and NAD^+-glycohydrolase reactions (Fig. 1 and Table 1).

Since NAD^+ and its analogs are known to have protective effects on the structure and activity of fragment A from diphtheria toxin (8), these results implied that NAD^+ might protect the enzymically active domain of exotoxin A from degradation. Figure 2 shows the band patterns obtained when toxin samples treated with chymotrypsin in the presence and absence of NAD^+ were electrophoresed on polyacrylamide gels in the presence of SDS. No band except that of chymotrypsin was observed with toxin that had been incubated in the absence of NAD^+ or with toxin treated at 100°C for 2 min before chymotryptic digestion in the presence of NAD^+. In contrast, the unheated toxin digested with chymotrypsin in the presence of NAD^+ showed an additional strong band at 26,000 daltons. When duplicate gels were fractionated and the proteins were eluted and assayed, a peak of ADP-ribosyltransferase activity was found at the position of the 26,000-dalton peptide in the NAD^+-treated sample; no activity was observed in the sample without NAD^+ (Fig. 3). In other

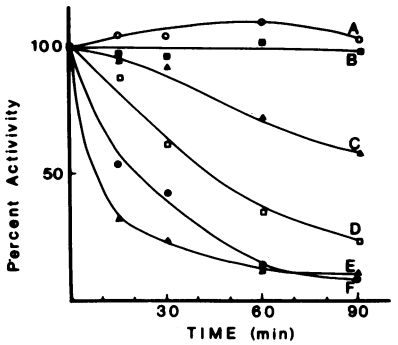

FIG. 1. *ADP-ribosyltransferase activity of reduced exotoxin A treated with chymotrypsin in the presence of NAD^+ and related compounds. A sample of exotoxin A was treated for 20 min with 5 mM DTT in the presence of 4 M urea. DTT and urea were removed by passage over a column (1.5 by 7 cm) of Sephadex G-50. The reduced, denatured exotoxin (0.4 mg/ml) in 50 mM Tris-hydrochloride buffer (pH 8.2)–1 mM EDTA was incubated with 50 μg of chymotrypsin per ml at 25°C in the presence of NAD^+ or various NAD^+ analogs in a 100-μl reaction mixture. At the times indicated, 10-μl samples were removed and diluted 1,000-fold in a solution of phenylmethylsulfonyl fluoride (5 μg/ml) in 50 mM Tris-hydrochloride (pH 8.2), and the ADP-ribosyltransferase activity was determined. Chymotrypsin treatment was in the presence of 15 mM NAD^+ (line A), 15 mM adenine (line B), 15 mM nicotinamide (line C), or 15 mM adenosine (line D). Line E, No analog present in the reaction mixture; line F, 15 mM ADP-ribose present.*

TABLE 1. *Inhibition constants of NAD^+ analogs in the ADP-ribosyltransferase and glycohydrolase reactions*

Compound	K_i by inhibition of initial rate of ADP ribosylation (μM)	K_i by inhibition of initial rate of NAD^+-glycohydrolase (μM)
Adenine	58	64
Nicotinamide	80	200
Adenosine	120	420
Nicotinamide mononucleotide	>1,000	>1,000
ADP-ribose	>2,000	>2,000
Adenosine 5′-triphosphate	>2,000	>2,000

experiments an additional band of stained material with an M_r of 25,000 was occasionally found in samples of toxin treated with chymotrypsin in the presence of NAD^+.

The active peptide derived by chymotryptic cleavage in the presence of NAD^+ was purified by chromatography, first on diethylaminoethyl cellulose and then on Sephacryl S-200. The purified peptide retained both NAD^+-glycohydrolase and ADP-ribosyltransferase activities, thus supporting the conclusion that the former activity was not due to a contaminating enzyme. We estimated that the 26,000-dalton fragment of exotoxin A is almost twice as active in catalyzing the hydrolysis of NAD^+ as the reduced, intact toxin.

The purified fragment bore other similarities to the fragment isolated from culture fluids. The reduced fragment contained less than 0.1 sulfhydryl group per molecule, as determined by reaction with radioactive iodoacetic acid, and at concentrations up to 1 nM it showed no inhibition of protein synthesis in HeLa or 3T3 cells during a 24-h incubation.

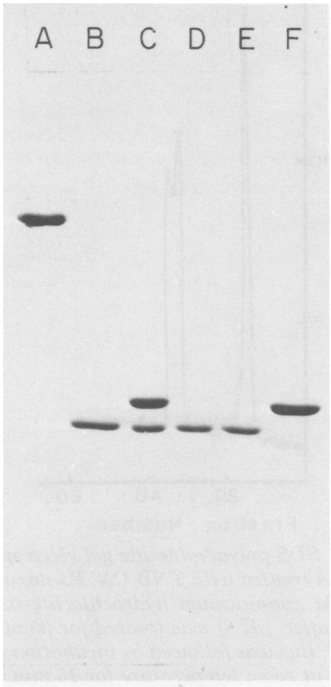

FIG. 2. *SDS-polyacrylamide gel pattern of chymotrypsin-treated exotoxin A. Thiol-activated exotoxin A (0.5 mg/ml) was treated with chymotrypsin (100 μg/ml) in the presence and absence of NAD^+ for 90 min at 25°C. The reaction was stopped by adding 10 μg of phenylmethylsulfonyl fluoride per ml and analyzed by SDS-polyacrylamide gel electrophoresis. Lane A, Exotoxin A incubated for 90 min in the absence of chymotrypsin and NAD^+; lane B, chymotrypsin alone; lane C, exotoxin A incubated in the presence of chymotrypsin and NAD^+; lane D, exotoxin A incubated in the presence of chymotrypsin; lane E, exotoxin A heated previously at 100°C for 2 min and then incubated in the presence of chymotrypsin and NAD^+; lane F, active peptide from culture fluids of P. aeruginosa, as described by Chung and Collier (2).*

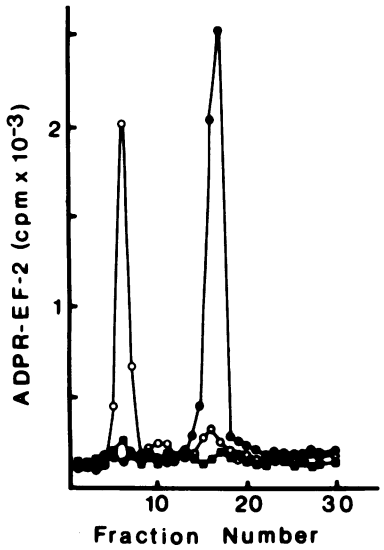

FIG. 3. *Profiles of ADP-ribosyltransferase (ADPR) activity eluted from SDS-polyacrylamide gels containing thiol-activated exotoxin A before and after treatment with chymotrypsin. Samples were treated with chymotrypsin and analyzed by SDS-polyacrylamide gel electrophoresis, as described in the legend to Fig. 2. After the conclusion of electrophoresis, lanes were cut out and divided into 2-mm slices. Individual slices were incubated in 1 ml of 20 mM Tris-hydrochloride buffer (pH 8.2)–1 mM EDTA (pH 8.2) for 12 h at 4°C. A sample from each fraction was then assayed for ADP-ribosyltransferase activity. Symbols: ○, exotoxin A; ●, exotoxin A treated with chymotrypsin in the presence of NAD^+; ■, exotoxin A treated with chymotrypsin in the absence of NAD^+. The direction of electrophoresis was from left to right. The peaks of enzymic activity of the lanes containing exotoxin A and the chymotryptic digest corresponded to M_r values of 66,000 and 26,000.*

Active fragments produced by chemical cleavage. Cleavage of exotoxin A at methionine residues with cyanogen bromide produced several peptides, most of which were insoluble in aqueous buffers. The predominant soluble species visible on SDS gels consisted of a double band corresponding to an M_r of 50,000. This was active in catalyzing the ADP ribosylation of EF-2 (Fig. 4). Assuming that none of the methionine residues is resistant to cleavage with CNBr, we conclude that all five methionines of the toxin are clustered within about one-third of the molecule and are completely absent from the domain carrying the catalytic center.

Two other enzymically active fragments (M_r,

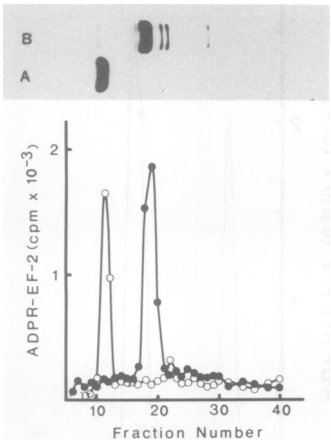

FIG. 4. *SDS-polyacrylamide gel electrophoresis of exotoxin A treated with cyanogen bromide. Exotoxin A was treated with cyanogen bromide as described in the text. Samples were analyzed by SDS-polyacrylamide gel electrophoresis in the presence of reducing agents. Duplicate samples were also analyzed for ADP-ribosyltransferase (ADPR) activity after elution of 2-mm slices in 1 ml of 20 mM Tris-hydrochloride buffer (pH 8.2)-1 mM EDTA. Lane A, exotoxin A (○); lane B, cyanogen bromide digest of exotoxin A (●). Electrophoresis was from left to right.*

30,000 and 32,000) were produced when reduced exotoxin A was treated with TNB-CN and subsequently incubated at an alkaline pH (Fig. 5). This procedure causes scission at cysteine residues. The appearance of two active fragments differing only slightly in molecular weight could result from incomplete cleavage at one of the half-cystines or from prior oxidation of one of the residues, making that residue unavailable for reaction with TNB-CN. The fragments were highly active in the NAD^+-glycohydrolase assay, as well as in ADP ribosylation of EF-2. These results imply that a 30,000-dalton segment representing approximately one-half of the toxin molecule and containing the catalytic center is devoid of half-cystine residues. This conclusion coincides with the finding that the 26,000-dalton active peptide isolated from culture fluids or generated by treatment of reduced whole toxin with chymotrypsin contains no half-cystines (2).

Reaction of reduced diphtheria toxin with TNB-CN yielded an enzymically active fragment of 20,000 daltons. This presumably corresponded to a fragment containing residues 1 to 185 of the toxin, which would be generated by cleavage at the single cysteine (residue 186) of the fragment A moiety. This is the shortest known enzymically active fragment from diphtheria toxin.

Activation of intact exotoxin A by partial reduction. As shown by Leppla et al. (12) and Vasil et al. (18), the activity of exotoxin A in catalyzing the ADP ribosylation of EF-2 is greatly enhanced by pretreatment of the toxin with reducing agents in the presence of urea. We studied the activation process by measuring ADP ribosylation of EF-2 after incubation of the toxin with varing concentrations of DTT for 15 min at 25°C in the presence of 4 M urea. As Fig. 6 shows, maximal activation was obtained with as little as 1 mM DTT.

In a second experiment we compared the ex-

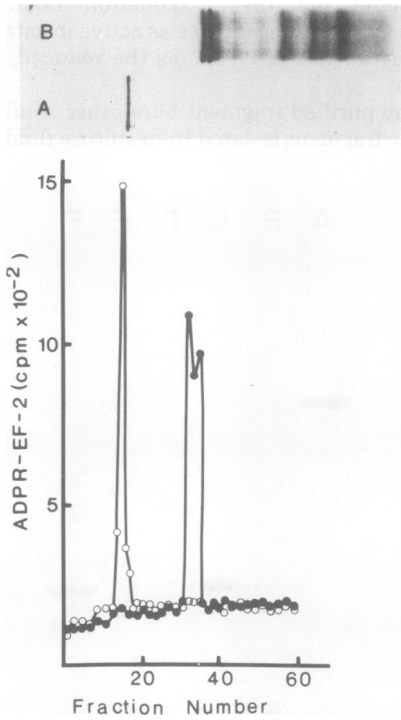

FIG. 5. *SDS-polyacrylamide gel electrophoresis of exotoxin A treated with TNB-CN. Exotoxin A (1 mg/ml in 6 M guanidinium hydrochloride-0.2 M Tris-acetate buffer, pH 8) was treated for 30 min with 10 mM DTT; this was followed by incubation with 0.1 M TNB-CN at room temperature for 15 min. The samples were then dialyzed against 50% acetic acid and lyophilized. For cleavage, the lyophilized preparation was dissolved in 6 M guanidinium hydrochloride in 0.1 M borate buffer (pH 9) and incubated at room temperature for 12 h. Guanidinium hydrochloride was removed by passage of toxin over a small column of Sephadex G-50, and samples were analyzed by SDS-polyacrylamide gel electrophoresis. The band pattern was determined by staining with Coomassie brilliant blue, and ADP-ribosyltransferase (ADPR) activity was assayed after elution of 1-mm slices in 0.5 ml of 20 mM Tris-hydrochloride buffer (pH 8.2)-1 mM EDTA. The direction of electrophoresis was from left to right. Lane A, exotoxin A (○); lane B, TNB-CN-treated exotoxin A (●).*

tent of reduction of disulfide bonds with the expression of ADP-ribosyltransferase activity. As Fig. 7 shows, maximal activity was manifested under conditions in which four free sulfhydryls were available for labeling with [^{3}H]iodoacetate. Thus, reduction of only two of the four disulfide bridges in the toxin appears to be sufficient for maximal expression of activity. DTT alone failed to activate the native toxin significantly, and no incorporation of label from [^{3}H]iodoacetate was detected under these conditions.

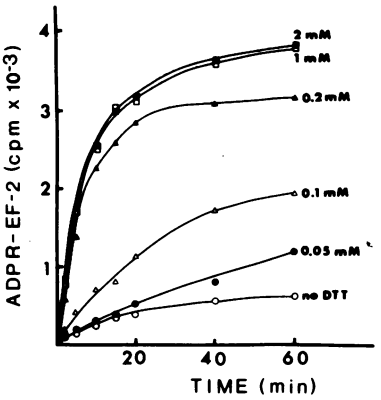

FIG. 6. *Kinetics of ADP ribosylation of EF-2 by thiol-activated exotoxin A. Samples of exotoxin A (30 μg/ml) were treated with 4 M urea and varing concentrations of DTT in 10 mM Tris-hydrochloride buffer (pH 8) for 15 min at 25°C. Toxin was then diluted 10-fold, and rates of ADP-ribosyltransferase (ADPR) activity were determined by removing small samples from the reaction mixture at regular time intervals and precipitating ADP-ribosyl EF-2 with trichloroacetic acid.*

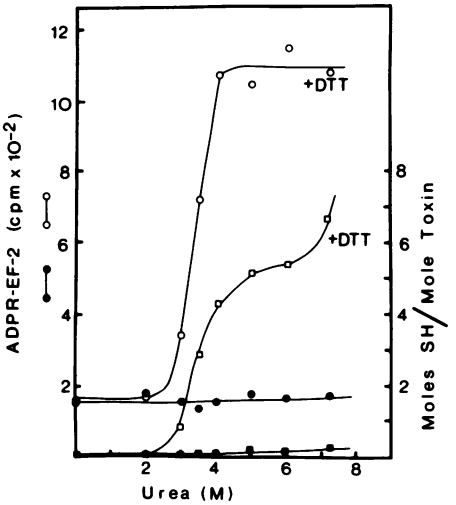

FIG. 7. *Dependence of ADP-ribosyltransferase (ADPR) activity on the extent of reduction of disulfide bonds of exotoxin A. Exotoxin A (0.2 mg/ml) was treated with varing concentrations of urea in the presence and absence of 1 mM DTT. Free SH groups were determined by reaction with [^{3}H]iodoacetic acid. ADP-ribosyltransferase activity was measured after 1,000-fold dilution into 50 mM Tris-hydrochloride buffer (pH 8.2) containing 0.2 mg of bovine serum albumin per ml.*

Enzymic properties of activated exotoxin A. K_m values for NAD$^+$ and EF-2 in the ADP ribosylation of EF-2 were determined by analyzing reciprocal plots of initial rates measured with toxin maximally activated by reduction. The K_m for NAD$^+$ was 2.5 μM, and that for EF-2 was 0.15 μM. These values are similar to those observed with both diphtheria toxin fragment A (1.4 and 0.15 μM, respectively [1]) and the 26,000-dalton active fragment of exotoxin A from culture fluids (6 and 0.15 μM [2]).

Exotoxin A activated by reduction also exhibited NAD$^+$-glycohydrolase activity. This activity has been found with diphtheria toxin fragment A and activated forms of cholera and *Escherichia coli* heat-labile enterotoxins, as well as the active fragments from exotoxin A. No appreciable NAD$^+$-glycohydrolase activity was detected in untreated exotoxin A. The conditions necessary for eliciting this activity in exotoxin A were identical to those required for the manifestation of ADP-ribosyltransferase activity.

The NAD$^+$-glycohydrolase reaction catalyzed by activated exotoxin A had a pH optimum of 7.8 and a temperature optimum of 37°C. The K_m for NAD$^+$ was 12 μM, compared with 8 μM measured for diphtheria toxin fragment A. Competitive inhibition of the NAD$^+$-glycohydrolase reaction of activated exotoxin A by NAD$^+$ analogs and related compounds showed a pattern similar to that observed with either the 26,000-dalton active fragment or diphtheria toxin fragment A. The thermal stability of activated exotoxin A was markedly different from that of diphtheria toxin fragment A, however. Treatment for 5 min at 60°C or above completely inactivated exotoxin A, whereas the diphtheria toxin fragment was only marginally affected.

DISCUSSION

We have described a method for producing a 26,000-dalton enzymically active peptide by treatment of reduced exotoxin A with chymotrypsin in the presence of NAD$^+$. This peptide appears to be identical to that isolated from culture fluids or stored toxin preparations, but may differ in minor details, such as the nature of the termini. The effect of NAD$^+$ is similar to the protective effects of substrates observed in many other enzyme systems, including diphthe-

ria toxin fragment A. The binding of NAD^+ may block access of chymotrypsin to sensitive bonds adjacent to the NAD^+ binding site or it may cause rearrangement of the tertiary structure of the 26,000-dalton domain carrying the catalytic center such that this entire domain becomes less susceptible to proteolytic attack. We were unable to generate the active fragment by chymotryptic treatment of native exotoxin A or reduced exotoxin A that had been heated at 100°C for 5 min.

Precisely how the 26,000-dalton active fragment is generated in culture fluids and purified toxin preparations is not known. Although bacterial proteases are presumably involved, the identity of the protease(s) involved is unclear. Since NAD^+ is absent from purified toxin preparations in which the fragment is sometimes generated, it must not be an obligatory requirement for stability of the fragment.

Further insight into structure-function relationships of exotoxin A was gained by studying active fragments derived by chemical treatment. Since the naturally occurring 26,000-dalton fragment is devoid of half-cystines (2), it was possible to generate larger enzymically active peptides (M_r, 30,000 and 32,000) by treatment of reduced exotoxin A with TNB-CN, a reagent that cleaves specifically at half-cystine residues. An even larger fragment (M_r, 50,000) was formed when exotoxin A was treated with cyanogen bromide. These results indicate that sulfur-containing amino acids are not important for activity of the 26,000-dalton domain carrying the catalytic center and imply that they are confined to approximately one-half the molecule. The location of the 26,000-dalton active fragment within exotoxin A is not known.

Exotoxin A is secreted in an enzymically inactive form. Activation may be effected without proteolysis by reduction of disulfide bonds in the presence of urea (12, 18). Careful titration revealed that approximately one-half of the disulfide bonds must be reduced for exotoxin A to exhibit maximal ADP-ribosyltransferase activity. The loss of cytotoxicity after treatment of exotoxin A with thiols may have resulted from a perturbation of the receptor-binding region of the molecule. Kinetic constants for the ADP-ribosyltransferase and NAD^+-glycohydrolase reactions catalyzed by reduced exotoxin A were similar to those determined for the 26,000-dalton peptide and diphtheria toxin fragment A. This supports the notion that diphtheria toxin and exotoxin A are homologous, at least in the domain carrying the catalytic center. Nevertheless, the enzymic activity of thiol-treated exotoxin A, like that of the 26,000-dalton peptide, was insensitive to anti-diphtheria toxin antibody or to anti-fragment A.

Relationships of enzymic activity to structure in exotoxin A show both similarities and differences to the relationships in diphtheria toxin. In both toxins maximal expression of activity in ADP-ribosylating EF-2 occurs only after covalent alterations involving fragmentation, reduction, or both (3). However, there are differences in the expression of NAD^+-glycohydrolase activity. Whereas native exotoxin A is apparently devoid of NAD^+-glycohydrolase activity, whole diphtheria toxin in either the intact form or the nicked form exhibits this activity in a magnitude comparable to the activity of its active fragment A (S. Lory, P. Bernard, and R. J. Collier, manuscript in preparation). Thus, a catalytically active NAD^+ binding site must be exposed on diphtheria toxin, whereas it is apparently obscured or distorted on exotoxin A. Although it has not yet been possible to generate from exotoxin A a receptor-binding fragment comparable to that of fragment B from diphtheria toxin, this may simply be a matter of finding the appropriate conditions of cleavage. Our results imply that the region of the molecule containing the half-cystine and methionine residues serves this purpose.

Studies described here and elsewhere imply that several toxins, including exotoxin A, must undergo covalent alterations before their ultimate target molecules can be attacked within the cell. However, little is known about the actual processing events that occur in vivo. Reduction of disulfide bridges would most likely occur within the reducing environment within the cell, but proteolytic processing could conceivably occur at any of several points after attachment to the cell surface receptor (or with some toxins, e.g. diphtheria toxin, even before attachment). The mechanisms by which toxins or their active fragments enter cells are difficult to probe, and at the present time we know little about the requisite events in movement across membranes. Questions concerning both processing and entry mechanisms constitute the major barriers to our understanding of the actions of exotoxin A and diphtheria toxin.

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