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Enzymatically Active Peptide from the Adenosine Diphosphate-Ribosylating Toxin of *Pseudomonas aeruginosa*

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A nontoxic peptide (molecular weight, 26,000), which is active in catalyzing the adenosine diphosphate (ADP)-ribosylation of elongation factor 2, has been isolated from the culture supernatant of *Pseudomonas aeruginosa* strain 103 in stationary phase. Like fragment A from diphtheria toxin, the active peptide catalyzed the hydrolysis of nicotinamide adenine dinucleotide as well as the ADP-ribosylation of elongation factor 2 and showed similarities to fragment A in specific activity, kinetic constants, pH optimum, and ionic sensitivity. These results provide strong evidence for a high degree of homology in the structures of their active sites. That the peptide is not identical to fragment A is shown by the fact that it was not neutralized by fragment A-specific antiserum and was different in amino acid composition and pH and thermal labilities. Although definitive evidence is lacking, there are data suggesting that this peptide is a proteolytic fragment from the ADP-ribosylating toxin (exotoxin A; molecular weight, 66,000) produced by the same strain of *P. aeruginosa*.

Among the toxic products excreted by *Pseudomonas aeruginosa* is a protein, termed exotoxin A by Liu (12), that is remarkably similar in action to diphtheria toxin. The pseudomonas toxin, like diphtheria toxin, is cytocidal for a number of cultured cell lines (17) and inhibits protein synthesis in these cells as well as in numerous organs in whole animals (18). Furthermore, and most importantly, both toxins apparently inhibit protein synthesis by the same biochemical mechanism, namely, by catalyzing the adenosine diphosphate (ADP)-ribosylation of elongation factor 2 (EF-2), with nicotinamide adenine dinucleotide (NAD) as the donor of ADP-ribose (8):

$$\text{EF-2} + \text{NAD} \rightleftarrows \text{ADP-ribose EF-2} + \text{nicotinamide} + \text{H}^+$$

Despite these similarities in action, the two toxins are not identical. There is no detectable cross-reactivity or cross-neutralization between the toxins, and they differ in size and amino acid composition (7, 11). Also, there are differences in species specificity. Whereas mouse cells are much more resistant than human cells to diphtheria toxin, the converse is true of the pseudomonas toxin (16, 17).

Another area in which comparison of the two toxins is of interest is structure-activity relationships. Diphtheria toxin is excreted as a proenzyme, consisting of a single polypeptide chain with a molecular weight of about 60,000. In this form it is toxic for animals and cell cultures but is inactive in catalyzing the ADP-ribosylation of EF-2. This enzymatic activty is manifested only after limited proteolytic cleavage, by trypsin or similar enzymes, and reduction and can be shown to be a property of the N-terminal fragment A (molecular weight, 21,145) thus generated or of certain larger fragments containing fragment A. The C-terminal fragment B (molecular weight, 39,000) has no enzymatic activity but is required for toxicity. There is good evidence now that fragment B is responsible for recognition of and binding to receptors on the surfaces of susceptible cells (3).

Structure-activity relationships in the pseudomonas ADP-ribosylating toxin are less well understood. This toxin also appears to be excreted as an intact polypeptide chain, of molecular weight of about 66,000 (7, 11). It has been reported that this intact molecule is enzymatically active and that the activity can be stimulated 20- to 50-fold by treatment with urea and dithiothreitol without altering the molecular weight of the toxin (11). On the other hand, we and others have observed lower-molecular-weight peptides with ADP-ribosylation activity in crude preparations or aged, purified preparations of the pseudomonas toxin (7, 11; Chung

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and Collier, unpublished observations). The relationships among these observations remain to be elucidated.

Here we report the isolation and characterization of a 26,000-dalton, enzymatically active peptide from the culture supernatants of *P. aeruginosa* strain 103. We have compared the peptide to fragment A from diphtheria toxin and have obtained evidence relating the peptide to the intact pseudomonas toxin.

MATERIALS AND METHODS

Bacteria. *P. aeruginosa* strain 103 (PA 103) was kindly provided by B. H. Iglewski. The organism was grown in the dialyzable fraction of Trypticase soy broth under high aeration. Cells in logarithmic growth were rapidly frozen in 10% glycerol and stored at −70°C as stock cultures.

Growth and toxin production. The medium used for the growth of PA 103 and production of toxin was essentially the same as reported by Liu (13). Trypticase soy broth (Baltimore Biological Laboratory, Cockeysville, Md.) was prepared at 10-fold normal concentration, and high-molecular-weight material was removed by chromatography on a Sephadex G-25 column (9.5 by 60 cm) equilibrated with water. The medium was sterilized in the concentrated form and diluted into sterile water to the suitable final concentration. At the time of inoculation, the medium was supplemented with 1% glycerol and 50 mM monosodium glutamate.

An overnight culture of PA 103 (200 ml) grown in the same medium was inoculated to an initial cell density of about 10^8 cells per ml into 40 liters of medium in a carboy, prewarmed to 32°C. Antifoam (SAG 471, Union Carbide Corp., New York, N.Y.) was added (final concentration, 10 μl/liter), and the culture was vigorously aerated with pure oxygen. After appropriate periods of incubation, cells were removed by centrifugation at 10,000 × *g* for 30 min. The culture supernatant was processed as described.

Crude EF-2 from wheat germ. Partially purified EF-2 from wheat germ was prepared by a procedure modified from Legocki and Marcus (10) and Seal et al. (21). Thirty grams of raw wheat germ was suspended in 240 ml of cold buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0), 50 mM magnesium acetate, 0.1 M KCl, 4 mM calcium chloride, 5 mM 2-mercaptoethanol, and 1 μg of phenylmethylsulfonyl fluoride per ml. The suspension was homogenized at top speed in a Waring blender for 50 s (five blendings, each of 10-s duration). Cell debris and ribosomes were removed by successive centrifugations at 21,000 × *g* for 15 min and 250,000 × *g* for 1 h. The postmicrosomal supernatant was fractionated with ammonium sulfate; material that precipitated between 30 and 50% saturation at 4°C contained approximately 70% of the total EF-2 content in the supernatant. The crude EF-2 was redissolved in approximately 1/20 the original volume of buffer containing 50 mM Tris-hydrochloride (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM 2-mercaptoethanol, and 1 μg of phenylmethylsulfonyl fluoride per ml and exhaustively dialyzed in the cold against the same buffer. Traces of precipitate were removed by centrifugation. Dithiothreitol was added to a concentration of 2 mM and glycerol was added to a concentration of 5%, and the preparation was stored at −70°C in small portions.

Purification of diphtheria toxin and its fragment A. Partially purified diphtheria toxin was purchased from Connaught Laboratories, Toronto, Canada. It was further purified as described previously (4). Conversion of diphtheria toxin to nicked toxin by limited trypsinization and separation of fragments A and B by gel filtration in the presence of 4 M urea were performed as reported elsewhere (Chung and Collier, manuscript in preparation).

Preparation of radioactive NAD. NAD labeled in the adenine portion of the molecule was synthesized from [adenine-^{14}C]adenosine triphosphate and nicotinamide mononucleotide as described previously (9). [Nicotinamide-^{14}C]NAD was purchased from Amersham/Searle Corp., Des Plaines, Ill. (specific activity, 59 mCi/mmol).

Alkylation with [^{3}H]NEM. One-half-nanomole samples of diphtheria toxin fragment A and pseudomonas active peptide in 30 μl of 5 mM sodium phosphate buffer, pH 8.0, were reduced at room temperature for 2 h with 65 nmol of dithiothreitol, with or without 8 M urea. The pH was then adjusted to 7.0 with 5 μl of 1 M sodium phosphate, pH 7.0, and 130 nmol of *N*-[^{3}H]ethylmaleimide([^{3}H]NEM) in 30 μl of pentane (New England Nuclear Corp., Boston, Mass.; 230 mCi/mmol) was added to the mixture and allowed to react for 1 min at room temperature under nitrogen. 2-Mercaptoethanol, 1 μmol (10 μl of a 0.1 M solution), was then added to stop further reaction. As a control, 2-mercaptoethanol was added to the samples before the addition of [^{3}H]NEM. A portion of the protein was removed from each sample and diluted into 0.1 ml of Tris-bovine serum albumin buffer (10 mM Tris-hydrochloride, pH 7.6, containing 0.1 mg of bovine serum albumin per ml); an equal volume of 10% cold trichloroacetic acid was added, and the precipitate was collected on glass-fiber filters (GF/C, Whatman, Inc., Clifton, N.J.). The extent of alkylation of NEM was measured by liquid scintillation counting (1). The remaining sample was diluted 1,000-fold in Tris-bovine serum albumin buffer, and ADP-ribosylation activity was assayed as described below.

ADP-ribosylation assays. ADP-ribosylation activity was measured by the incorporation of radioactivity from [adenine-^{14}C]NAD into trichloroacetic acid-precipitable material in the presence of crude EF-2. The reaction was performed at 25°C in 0.1 ml of 10 mM Tris-hydrochloride, pH 8.0, and 1 mM EDTA. Partially purified EF-2 from wheat germ and [adenine-^{14}C]NAD were present at 0.15 and 0.36 μM, respectively. Trichloroacetic acid precipitation on paper was performed as modified from Bollum (2). Whatman 3MM paper was ruled into 1-inch (2.54-cm) squares, impregnated with trichloroacetic acid by immersion in ether containing 10% trichloroacetic acid, and subsequently dried in air. ADP-ribosylation was stopped by spotting 50 μl from a

reaction mixture onto a square. After all samples were collected, the paper was immersed and gently agitated for 10 min in 5% trichloroacetic acid in a glass tray. Trichloroacetic acid-soluble material was removed by repeating the washing procedure once. The paper was similarly washed once with acetone and air-dried. Individual squares were cut and counted in 10 ml of scintillation fluid containing 0.7% 2,5-diphenyloxazole and 0.005% 1,4-bis-[2]-(5-phenyloxazolyl)benzene in toluene.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis under nondenaturing conditions was performed as described by Maizel (15). Electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) was performed in a slab gel apparatus modified from Reid and Bieleski (20) with a discontinuous SDS-polyacrylamide stacking system, as described by Maizel (15).

RESULTS

Occurrence of the active peptide. Late in the stationary phase of the growth of PA 103, low levels of ADP-ribosylation activity were observed in the culture medium. The activity began to appear as cells entered stationary phase and increased steadily for about 4 h before becoming constant (Fig. 1). The culture supernatant, at the time of maximal activity, was concentrated by ultrafiltration with an Amicon PM-10 membrane. When the concentrate was fractionated on a Sephadex G-150 column, two peaks of ADP-ribosylation activity, corresponding to about 66,000 and 26,000 daltons, were observed (Fig. 2). The high-molecular-weight peak corresponds in molecular weight and enzymatic activity to the pseudomonas ADP-ribosylating toxin previously described (7, 11). The low-molecular-weight peak may correspond to one of the low-molecular-weight active peptides eluted from SDS-gels by Leppla (11). The amount of activity associated with the high-molecular-weight peak varied from preparation to preparation and was entirely absent in some cases.

Purification of the enzymatically active, 26,000-dalton peptide. Cell-free culture supernatant was concentrated by precipitation with 50 mM zinc acetate (14). The precipitate was collected by low-speed centrifugation and redissolved in 0.3 M sodium citrate, pH 8.0, to approximately 1/20 the original volume. Diisopropyl fluorophosphonate and phenylmethylsulfonyl fluoride were added, each to a final concentration of 1 μg/ml. After insoluble material was removed by centrifugation, the preparation was fractionated with ammonium sulfate at 4°C. Material that precipitated from 40 to 60% saturation was collected and redissolved in 10 mM Tris-hydrochloride, pH 7.8, containing 1 mM EDTA and 1 μg of phenylmethylsulfonyl fluoride per ml and was then extensively dialyzed in the cold against the same buffer.

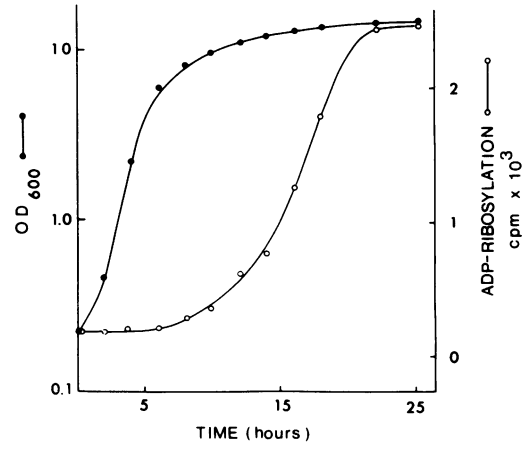

FIG. 1. *Time course of appearance of ADP-ribosylation activity. The growth of PA 103 at 32°C in a small flask on a rotary shaker was followed by measuring the optical density at 600 nm (OD_{600}). Samples of the culture supernatant, taken at various intervals, were desalted on a small Sephadex G-25 column (0.9 by 6 cm, equilibrated with 10 mM Tris-hydrochloride, pH 7.6, and 1 mM EDTA), and the desalted samples were assayed for ADP-ribosylation activity as described in the text.*

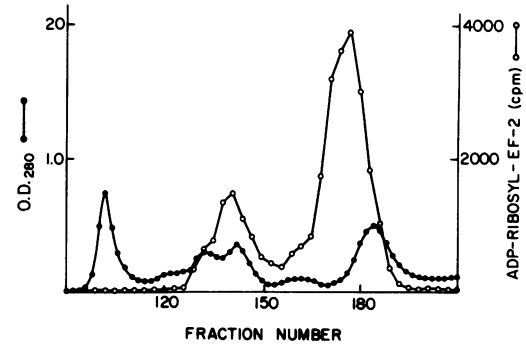

FIG. 2. *Chromatography of concentrated culture supernatant on Sephadex G-150. The culture supernatant (200 ml) of a 24-h culture of PA 103 was concentrated to about 7 ml by ultrafiltration with an Amicon PM-10 membrane and further concentrated by dehydration from outside a dialysis bag with dry Sephadex G-50. The concentrated culture supernatant was fractionated on a Sephadex G-150 column (1.5 by 70 cm) equilibrated in 10 mM Tris-hydrochloride, pH 7.6, and 1 mM EDTA. Fractions of 0.7 ml were collected at a rate of 3 ml/h. Protein concentration was monitored by absorbance at 280 nm, and ADP-ribosylation activity was measured as described in the text.*

Crude material was chromatographed on a diethylaminoethyl-cellulose column (Bio-Rad, Cellex-D, 3.8 by 20 cm) equilibrated with buffer

A (10 mM Tris-hydrochloride, pH 7.8, and 1 mM EDTA). The column was washed with 3 bed volumes of buffer A, and the active peptide was eluted with a linear salt gradient, 0 to 0.6 M NaCl in buffer A. The peptide eluted at about 0.15 M NaCl. Peak fractions were pooled, concentrated by ultrafiltration in an Amicon concentrator with a PM-10 membrane, and dialyzed overnight against buffer B (20 mM Tris-hydrochloride [pH 7.0], 1 mM EDTA, and 1 μg of phenylmethylsulfonyl fluoride per ml). The material was then chromatographed on a second anion-exchange column, QAE-Sephadex A-25 (2.5 by 17 cm), equilibrated with buffer B. The active peptide was eluted with a linear salt gradient, 0 to 0.7 M NaCl in buffer B, and then concentrated by precipitation in 90% saturated ammonium sulfate. The precipitate was redissolved in 1 ml of buffer B and chromatographed on a Sephadex G-75 column (1.2 by 75 cm) equilibrated with buffer B.

The peptide was further purified on a hydroxyapatite column (Bio-Rad HT, 1.7 by 7 cm). Peak fractions from the Sephadex G-75 column were dialyzed against buffer C (5 mM sodium phosphate buffer, pH 6.9) and applied to the column. Extensive washing removed all unadsorbed material, and the active peptide was eluted with a linear sodium phosphate gradient, 5 mM to 0.4 M in buffer C. The eluted peptide contained one major contaminating protein, which was resolved at pH 6.6 on a diethylaminoethyl-Sephadex A-25 column eluted with a shallow NaCl gradient, 0 to 0.4 M, in 10 mM Tris-hydrochloride, pH 6.6. Fractions under the activity peak were analyzed on SDS-polyacrylamide gels, and only the fractions that appeared pure were pooled. Figure 3 shows the purity of the active peptide at each stage of purification.

Proof of ADP-ribosyl transfer. We have performed the following experiments to prove that the reaction catalyzed by the active peptide from PA 103 supernatants is indeed ADP-ribosylation of EF-2.

(i) Samples of diphtheria toxin fragment A or the pseudomonas active peptide were incubated with crude EF-2 and [adenine-^{14}C]NAD, and a portion of each reaction mixture was subjected to electrophoresis in a discontinuous 10% slab polyacrylamide gel. The stained gel slab was dried onto filter paper under vacuum and exposed to X-ray film to detect covalently labeled protein bands. A single band (molecular weight, 100,000) was labeled in both cases, indicating EF-2 as the common target protein (Fig. 4). Antiserum against diphtheria toxin fragment A completely blocked the activity of fragment A but had no effect on the activity of the pseudomonas active peptide.

(ii) To determine whether the radioactivity transferred to EF-2 by the pseudomonas active

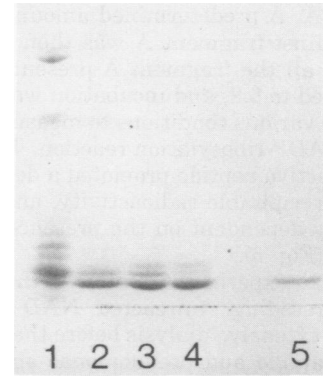

FIG. 3. *SDS-polyacrylamide gel electrophoresis of the active peptide at various stages of purification. The gel showed purity of the peptide after chromatography on (1) diethylaminoethyl-cellulose, (2) QAE-Sephadex A-25, (3) Sephadex G-75, (4) hydroxyapatite, and (5) diethylaminoethyl-Sephadex A-25.*

FIG. 4. *Autoradiographic identification of covalently labeled protein bands. Crude wheat germ EF-2, in the presence of [adenine-^{14}C]NAD, was incubated with (1) fragment A from diphtheria toxin, (2) fragment A and anti-fragment A serum, (3) pseudomonas active peptide, and (4) pseudomonas active peptide and anti-fragment A serum. A sample from each incubation mixture was analyzed in a 10% slab polyacrylamide gel in the presence of 0.1% SDS. The stained gel was dried under vacuum onto filter paper and exposed to X-ray film for the location of radioactively labeled protein bands.*

peptide represented ADP-ribose, we took advantage of the reversibility of the ADP-ribosylation reaction. Crude [^{14}C]ADP-ribose EF-2 was prepared by reacting EF-2 with [adenine-^{14}C]NAD in the presence of diphtheria toxin fragment A. A predetermined amount of antiserum against fragment A was then added to inactivate all the fragment A present; the pH was lowered to 6.2, and incubation was continued under various conditions to measure reversal of the ADP-ribosylation reaction. The pseudomonas active peptide promoted a decrease in the acid-precipitable radioactivity, and the decrease was dependent on the presence of nicotinamide (Fig. 5).

(iii) In an experiment similar to that immediately preceding, unreacted NAD was removed by extensive dialysis before the addition of nicotinamide and pseudomonas active peptide. The acid-soluble radioactivity subsequently released was identified as [^{14}C]NAD by thin-layer chromatography on polyethyleneimine-cellulose (Fig. 6) (19).

The results under (ii) and (iii) demonstrated clearly that the pseudomonas active peptide can catalyze reversal of the forward reaction catalyzed by diphtheria toxin fragment A. These experiments provide compelling evidence that the pseudomonas active peptide catalyzes the same reaction with the same substrate and site specificity as diphtheria toxin fragment A. Similar experiments have been performed with intact exotoxin A by Iglewski and co-workers (7; personal communication).

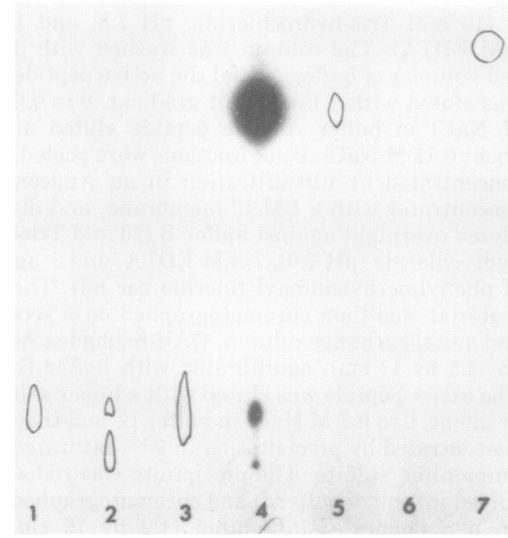

FIG. 6. *Autoradiographic identification of released radioactivity after reversal of ADP-ribosylation by pseudomonas active peptide. The trichloroacetic acid-soluble fraction was extracted with water-saturated ethyl acetate to remove nicotinamide and was lyophilized. The residue was dissolved in 20 μl of water and analyzed on a polyethyleneimine-cellulose thin-layer plate with marker compounds. The positions of radioactive samples were located by autoradiography. (1) Adenosine monophosphate marker, (2) ADP marker, (3) ADP-ribose marker, (4) radioactive material released after reversal, (5) NAD marker, (6) acid-insoluble material prior to reversal, (7) nicotinamide marker.*

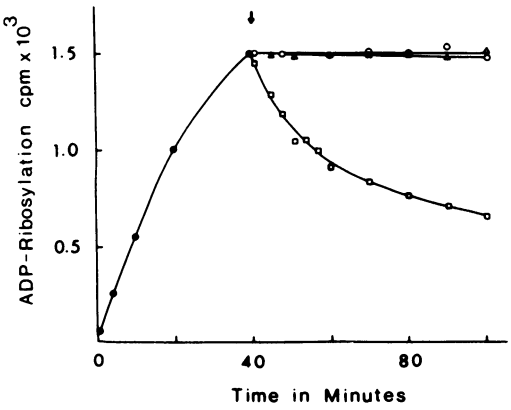

FIG. 5. *Reversal of ADP-ribosylation of EF-2 by pseudomonas active peptide. Wheat germ EF-2 was ADP-ribosylated by fragment A from diphtheria toxin for 40 min, at which time a predetermined amount of anti-fragment A serum was added, and the pH of the reaction mixture was adjusted to 6.2 by acetic acid. The reaction mixture was divided into several portions, and reversal was followed with additions of (i) 0.5 μM pseudomonas active peptide* (○), *(ii) 0.1 M nicotinamide* (△), *and (iii) 0.5 μM pseudomonas active peptide and 0.1 M nicotinamide* (□).

General properties of the active peptide. When subjected to electrophoresis in 10% discontinuous slab polyacrylamide gel in the presence of 0.1% SDS, the purified peptide migrated as a single polypeptide chain of about 25,000 daltons. Its molecular weight was unchanged by prior reduction with 2-mercaptoethanol. Part of the ADP-ribosylation activity could be recovered from SDS-gel slices, indicating the ability of a portion of the molecules to refold into an enzymatically active form after dilution of the SDS. We have noticed that wheat germ EF-2 was unsuitable for assaying the active peptide when the latter was recovered from SDS-gels. In cases when such assays were performed, partially purified reticulocyte EF-2 was used instead. We believe this anomaly is due to enhanced inactivation of the active peptide, for example, by proteolytic degradation, after elution from SDS-gels. Fragment A from diphtheria toxin does not show such anomalous behavior.

The peptide also appeared as a single protein band with an isoelectric point of about 5.3 when subjected to isoelectric focusing in 6% polyacrylamide gel.

Concentrations of the peptide as high as 1 μg/ml failed to inhibit protein synthesis in HeLa cells over a period of 18 h. For comparison, concentrations of diphtheria toxin of 1 ng/ml produce a 50% inhibition of protein synthesis under the same conditions.

ADP-ribosylation activity of the peptide showed a broad pH optimum, with maximal activity around pH 8.0. Like diphtheria toxin fragment A, the peptide showed no requirements for specific anions or cations and was relatively sensitive to ionic strength; for example, the presence of 0.1 M KCl or Tris-hydrochloride caused a 50% reduction in the initial rate of ADP-ribosylation.

The peptide was most stable at neutral pH and remained in solution when incubated in buffer with pH values between 3 and 11. The activity, assayed after diluting into neutral pH buffer, was relatively stable to extreme pH ranges but was less stable than that of diphtheria toxin fragment A (Fig. 7).

In contrast to diphtheria toxin fragment A, the active peptide was relatively sensitive to heat. Almost all enzymatic activity was lost after exposure to temperatures above 60°C for 5 min (Fig. 8).

Thiols had no effect on the enzymatic activity of the peptide. Reduction with 40 mM dithiothreitol and subsequent assay in the presence of 0.1 M 2-mercaptoethanol did not cause significant change in its enzymatic activity.

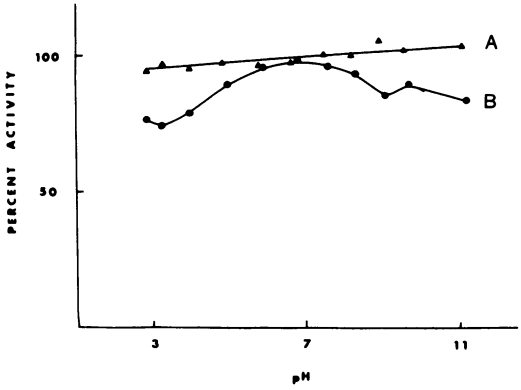

FIG. 7. *Comparison of pH stability of the pseudomonas active peptide to that of fragment A from diphtheria toxin. Both peptides (0.1 mg/ml) were incubated in a universal buffer (citrate, phosphate, borate, and diethylbarbiturate) adjusted to pH values between 3 and 11 with sodium hydroxide. After incubation at room temperature for 2 h, the samples were diluted 1,000-fold in 10 mM Tris-hydrochloride, pH 7.6, containing 0.1 mg of bovine serum albumin per ml, and enzymatic activities were determined as described in the text. (A) Fragment A, (B) pseudomonas active peptide.*

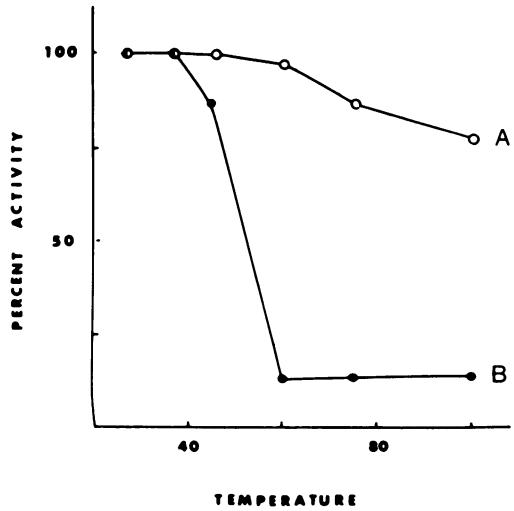

FIG. 8. *Comparison of thermal stability of the pseudomonas active peptide and fragment A from diphtheria toxin. Samples of (A) fragment A (0.75 mg/ml) and (B) the pseudomonas active peptide (0.6 mg/ml), in 10 mM Tris-hydrochloride, pH 7.6, were incubated for 5 min at the indicated temperatures. After diluting 1,000-fold in 10 mM Tris-hydrochloride, pH 7.6, containing 0.1 mg of bovine serum albumin per ml, samples were assayed for ADP-ribosylation activity.*

The susceptibility to alkylation with [^{3}H]-NEM was studied with reduced and unfolded active peptide. Little covalent modification and no significant loss in enzymatic activity were observed (Table 1). The low level of labeling, 0.1 mol/mol of peptide, can probably be attributed to nonspecific reaction of NEM with amino groups (22). Under the same conditions, diphtheria toxin fragment A was covalently modified to an extent of 0.9 mol/mol of protein, in close agreement with the fact that each molecule has a single free cysteine (4, 5). These results indicate that the pseudomonas active peptide possesses no free cysteine or internal disulfide linkages, a finding consistent with the observed insensitivity of the molecule to reducing agents.

Chemical properties. Amino acid analysis of the active peptide was performed according to Glazer et al. (6). As shown in Table 2, the peptide contained high quantities of acidic residues, in agreement with its low isoelectric point. The extinction coefficient, $E^{1\%}_{280}$, calculated from the total micromoles of amino acids recovered, is 15.6, whereas that of diphtheria

TABLE 1. *Modification of pseudomonas active peptide and diphtheria toxin fragment A with [³H]NEM*

Peptide	Extent of modification	
	Residue (mol/mol)	Enzymatic activity (%)
Pseudomonas active peptide	0.01	90
Control	0.01	100
Pseudomonas active peptide + urea	0.01	88
Control	0.01	98
Pseudomonas active peptide + dithiothreitol + urea	0.11	84
Control	0.03	82
Fragment A	0.03	100
Control	0.02	100
Fragment A + urea	0.11	92
Control	0.03	80
Fragment A + dithiothreitol + urea	0.9	87
Control	0.3	88

TABLE 2. *Comparison of amino acid compositions of pseudomonas active peptide and diphtheria toxin fragment A*

Residue	No. per molecule	
	Fragment A[a]	Pseudomonas active peptide
Lys	16	4
His	1	4
Arg	6 (7)	17
Asx	23	23
Thr	8	12
Ser	17	16
Glx	23	27
Pro	6	22
Gly	20	28
Ala	14	26
Val	16	13
Met	4	1
Ile	6	11
Leu	12	24
Tyr	10	10
Phe	7	8
Cys	1	0
Trp	2	ND[b]

[a] Data from reference 5.
[b] ND, Not determined.

toxin fragment A is 15.2, in good correlation with their similar phenylalanine and tyrosine contents. No cysteine or cystine was found, which was consistent with the lack of reactivity with NEM. It has been reported that pseudomonas ADP-ribosylating toxin has an unusually high arginine-to-lysine ratio (11), and it is clear that this ratio is conserved in the active peptide. The minimal molecular weight is 26,200, and the amino acid composition showed marked differences from that of diphtheria toxin fragment A. The significance of the exceptionally high proline content is unclear.

Catalytic properties of the active peptide. Like diphtheria toxin fragment A, the active peptide possesses a low NAD-glycohydrolase activity in addition to ADP-ribosylation. On a molar basis, the pseudomonas active peptide showed, within experimental error, specific activities in both ADP-ribosylation and NAD-glycohydrolase identical to those of diphtheria toxin fragment A.

Steady-state kinetic measurements of the ADP-ribosylation reaction catalyzed by the active peptide showed an intersecting pattern in double-reciprocal plots (Fig. 9), which is suggestive of a sequential enzymatic mechanism. The Michaelis constants, obtained by secondary extrapolations, were 0.15 and 8 μM for EF-2 and NAD, respectively. The initial rate pattern and the magnitude of the Michaelis constants were similar to those of diphtheria toxin fragment A (Chung and Collier, manuscript in preparation).

Concentrations of various inhibitors required

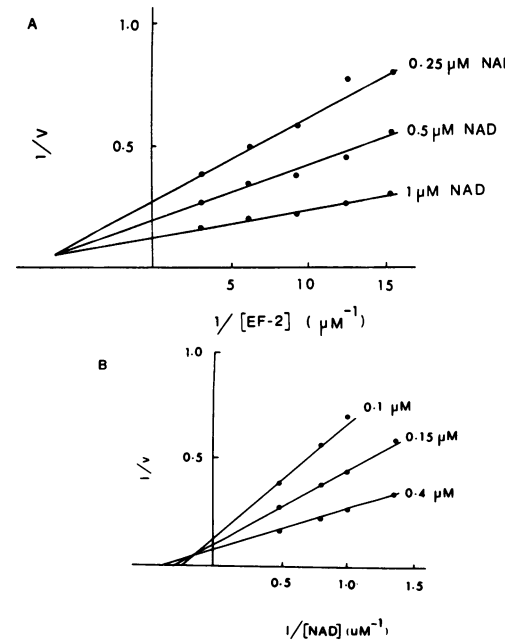

FIG. 9. *(A) Relationship between initial velocity and EF-2 concentration at selected constant NAD concentrations. The initial velocity is in picomoles of ADP-ribose EF-2 formed per 15 min at 25°C. (B) Relationship between initial velocity and NAD concentration at selected constant EF-2 concentrations.*

to produce half-maximal inhibition of ADP-ribosylation were determined with the pseudomonas active peptide or diphtheria toxin fragment A as enzymes. The similarities in K_i values for both enzymes are evident from the data shown in Table 3. This implies a strong similarity in the NAD-binding site on the two enzymes.

We also observed a weak quenching of the intrinsic tryptophan fluorescence of the active peptide (excitation, 290 nm; emission, 340 nm) upon adding NAD. Although the maximal reduction of fluorescence was only about 20%, the extent of quenching was a function of the concentration of NAD (Fig. 10). The concentration that caused half-maximal quenching was about 9 μM, which correlated well with the K_m of 8 μM for NAD in the ADP-ribosylation reaction.

Relationship between the active peptide and the pseudomonas ADP-ribosylating toxin. We have assumed that the active peptide under study here is produced by proteolytic cleavage of pseudomonas toxin, as fragment A is produced from diphtheria toxin. Although we have been unable to generate the peptide by treatment of pseudomonas toxin with trypsin, chymotrypsin, subtilisin, Pronase, or pancreatic elastase, there are some observations that support our assumption.

First, we have frequently observed degradation of pseudomonas toxin into smaller peptides during storage at −20°C. This breakdown is generally accompanied by an increase in total enzymatic activity. Additions of phenylsulfonyl fluoride, diisopropyl fluorophosphonate, and EDTA to crude toxin preparations reduce the degradation. Among the breakdown products are at least two peptides, which possess ADP-ribosylation activity, assayable after elution from SDS-gel slices. The smaller of the two peptides, which appears to correspond to the active peptide we report here, has a higher specific activity than the other peptide, which is about 1,500 daltons larger.

TABLE 3. *Inhibition constants of adenine- and nicotinamide-containing compounds and ADP-ribosylation of EF-2 catalyzed by diphtheria toxin fragment A and pseudomonas active peptide*

Compound[a]	K_i (μM)	
	Fragment A	Pseudomonas active peptide
Adenine	38	50
Adenosine	320	200
AMP	>3,000	>6,000
ADP, ATP	>3,000	>6,000
ADP-ribose	>3,000	>6,000
Nicotinamide	200	100
NMN	>3,000	>2,800

[a] AMP, Adenosine monophosphate; ATP, adenosine triphosphate; NMN, nicotinamide mononucleotide.

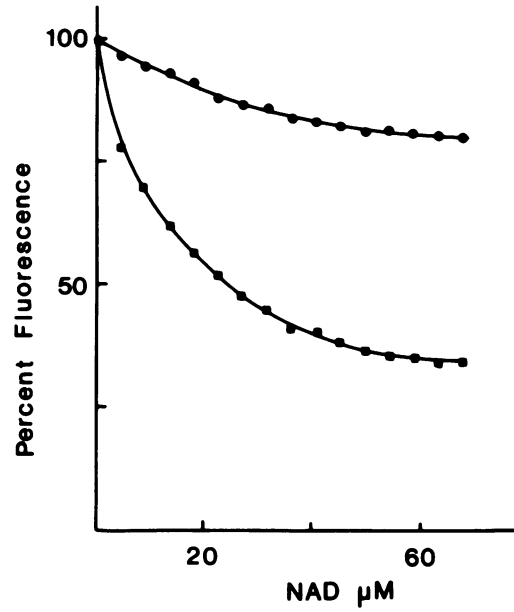

FIG. 10. *Dependence of the intrinsic tryptophan fluorescence of pseudomonas active peptide (●) and fragment A from diphtheria toxin (■) on NAD concentrations. Small quantities of NAD were added to solutions of the pseudomonas active peptide and fragment A (both at 1 μM), and the intensities of fluorescence (excitation, 285 nm; emission, 340 nm) were measured after each addition. A tryptophan solution of comparable fluorescence intensity was similarly titrated to correct for dilution and beam attenuation.*

Second, when a ribosome-free extract from PA 103 containing appreciable amounts of toxin was incubated at 37°C, we observed a shift in the size of the enzymatic activity over a period of 24 h from the 70,000-dalton range to a lower-molecular-weight range, on the order of 25,000 to 30,000. The extract was prepared by disrupting and removing large debris and ribosomes by centrifugation. Samples were taken after incubation for various periods at 37°C, each was chromatographed on a column of Sephadex G-150, and the profile of enzymatic activity in the collected fractions for each sample was determined.

Is the whole toxin molecule enzymatically active? Although it has been reported that pseudomonas toxin itself is enzymatically active (8, 11), it remains possible from the published data that the observed activity is in fact a property of peptides generated during the assay by cleavage of inactive toxin. We have

done two experiments to test this possibility.

First, the rate of ADP-ribosylation catalyzed by pseudomonas toxin, followed at 1-min intervals, was linear with time, with no observable lag. This is inconsistent with a time-dependent enzyme activation in situ, in which case a progressive increase in specific activity would have been observed. The experiment, however, did not rule out the possibility that maximal activation might have occurred within the first 15 s, i.e., before the removal of the first sample.

Second, we incubated pseudomonas toxin with crude wheat germ EF-2 under conditions identical to those of the normal ADP-ribosylation assay and subsequently fractionated the mixture on a Sephadex G-150 column. The position where ADP-ribosylation activity eluted remained at about 66,000 daltons, indicating that no molecular-weight change occurred during the incubation. The activity observed with pseudomonas toxin was therefore associated with the 66,000-dalton polypeptide, which is consistent with the conclusion reported by other workers (8, 11).

DISCUSSION

The enzymatically active peptide described in this report shows striking similarities in catalytic properties to those of fragment A from diphtheria toxin. The pseudomonas and diphtherial peptides have virtually identical specific activities in both ADP-ribosylation and NAD-glycohydrolase reactions, comparable Michaelis constants for NAD and EF-2, and similar sensitivities to inhibition by adenine- and nicotinamide-containing analogues of NAD. Furthermore, evidence from kinetic studies indicates that both peptides catalyze the ADP-ribosylation reaction by a sequential mechanism. These results all suggest a high degree of homology in the structures of the active sites on the two peptides.

Despite these similarities, there is clear evidence that the structures of the pseudomonas active peptide and fragment A are not identical. Differences in amino acid composition, pH and thermal stabilities, and lack of immunological cross-reactivity have been documented. Assuming that the two peptides are homologous and derived from a common ancestral protein, it appears that the active site has been conserved, with changes having occurred in the superstructure surrounding the site.

Although definitive evidence is not yet available, it is likely that the enzymatically active peptide from PA 103, like fragment A from diphtheria toxin, is produced by proteolytic action on the ADP-ribosylating toxin. The pseudomonas protease or proteases involved must differ in specificity from those which cleave diphtheria toxin to yield fragment A, but we have no further information at present about their identities. Assuming that the active pseudomonas peptide is indeed a fragment from the whole toxin, it is clear from the absence of half-cystine residues that it cannot be linked by disulfide bridges to the complementary portion of the toxin. We assume, from the lack of toxicity of the enzymatically active peptide, that the complementary portion of the toxin must contain a binding site for some cell surface receptor that mediates attachment and/or transmembrane transport of the toxin.

Leppla has recently reported that the ADP-ribosylating toxin from PA 103 is excreted in an enzymatically inactive form, with a molecular weight of 66,000, containing four disulfide bridges. Treatment of the toxin with urea and dithiothreitol apparently unmasks the active site without alterating the molecular weight of the molecule (11). Assuming that the enzymatically active peptide we have isolated represents a proteolytic fragment from the toxin, then proteolysis must represent a second pathway by which the enzymatic activity of the toxin may be manifested. No information is available at present to indicate which pathway may be operative in vivo. One can conceive that structural rearrangements similar to those presumably produced in the presence of urea plus dithiothreitol might occur during or after the entry into cells, as could proteolysis.

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