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THE EXOTOXIN OF P. AERUGINOSA: A PROENZYME HAVING AN UNUSUAL MODE OF ACTIVATION

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Summary

The exotoxin of Pseudomonas aeruginosa is a proenzyme possessing latent adenosine diphosphate ribose-transferase activity. Conversion to the active form can be effected by simultaneous treatment with a protein denaturant and a chemical able to split disulfide bonds. Activation results from a conformational change that exposes the previously buried active site. Proteolysis is not required.

The gram-negative bacterium Pseudomonas aeruginosa secretes a potent protein exotoxin which is lethal to a variety of experimental animals and cultured mammalian cells (1-3). This Pseudomonas exotoxin (PE)*can be produced in quantities of a few hundred milligrams and purified to a state near homogeneity (4,5). Iglewski and Kabat (6) showed that PE has the same enzymatic activity as diphtheria toxin (DE). Both toxins catalyze transfer of the adenosine diphosphate ribose (ADPR) portion of nicotinamide adenine dinucleotide (NAD) to eukaryotic elongation factor 2 (EF-2). Modified EF-2 is unable to perform its normal translocase function in protein synthesis, so toxin-exposed cells die. One characteristic feature of diphtheria toxin is its biosynthesis as an inactive proenzyme [reviewed by R. J. Collier (7)], which is rendered enzymatically active by cleavage into two large peptides, fragments A and B, through a process requiring peptide bond scission and reduction of a disulfide bridge. We show here that PE, like DE, is synthesized as a proenzyme,

*Abbreviations: PE, Pseudomonas exotoxin; DE, diphtheria toxin; DTT, dithiothreitol; ADPR, adenosine diphosphate ribose; GuHCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate.

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but that its activation can occur through a nonproteolytic process for which few precedents exist.

Methods

The Pseudomonas exotoxin was purified as described previously (5). Assay of ADPR-transferase activity was by incubation of [^{14}C]NAD (271 μCi/μmol, uniformly labeled in adenine, Amersham/Searle) with toxin and EF-2 as described (5) except that dithiothreitol (DTT) was omitted. EF-2 was obtained from a wheat germ homogenate as the 30-50% saturated ammonium sulfate fraction. It was chromatographed on DE-52 cellulose (Whatman) and treated with 2 mM iodoacetamide, which caused no decrease in its ADPR acceptor activity and eliminated the need for reducing agents in the ADPR transferase assay. Electrophoretic analyses and determinations of amino acid composition, N-terminal amino acids, and sulfhydryls followed methods used previously (5). Proteins were labeled by reductive methylation [modification of method of Means and Feeney (8)] with [^{3}H]- or [^{14}C]-labeled formaldehyde and sodium cyanoborohydride (manuscript in preparation).

Hydrolysis of [carbonyl-^{14}C]NAD (53 mCi/mmol, Amersham/Searle) was measured by extraction of the [^{14}C]nicotinamide into ethyl acetate (9). Samples (100 μl) containing 75 mM tris hydrochloride, 4 μM [^{14}C]NAD, and toxin were incubated 4 h at 37° and then mixed with 200 μl H_2O and 1.0 ml H_2O-saturated ethyl acetate. After vortexing 30 sec, 0.70 ml of the upper phase was transferred to 3.0 ml Aquasol (New England Nuclear) for scintillation counting.

Results and Discussion

Purified PE has a low level of ADPR-transferase activity which is not significantly increased by pretreatment with high concentrations of either DTT or urea alone (Fig. 1). However, simultaneous exposure to DTT and urea induces a large increase in activity, approximately 100-fold in the sample illustrated. Chemicals other than urea and DTT can also activate PE; increase of enzymatic activity equal to that shown in Fig. 1 is obtained when PE is treated simultaneously with any one of a number of protein denaturants [urea, guanidine hydrochloride (GuHCl), sodium dodecyl sulfate (SDS)] and a reagent capable of breaking disulfide bonds (DTT, cysteine, 2-mercaptoethanol, sulfite). These data demonstrate that PE is synthesized in an enzymatically inactive, proenzyme form.

We next asked whether activation involved a fragmentation of the polypeptide like that required for activation of DE or whether a disruption of the secondary and tertiary structure was sufficient. In previously reported analyses PE was shown to migrate as a single band of molecular weight 66,000 on

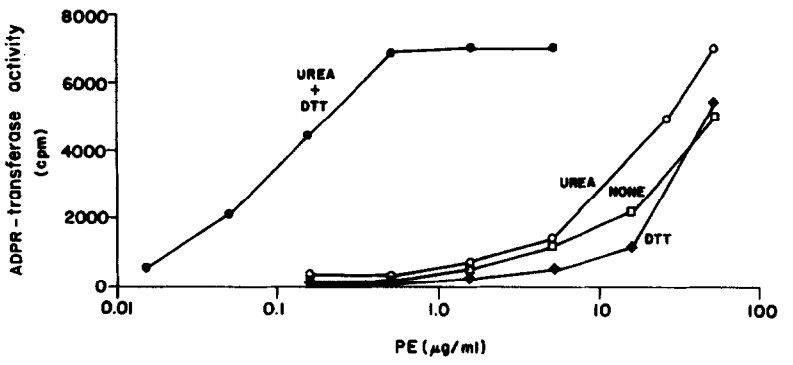

Figure 1. Effect of reduction and denaturation on ADPR-transferase activity of Pseudomonas exotoxin. Aliquots (5 μl) of a 7 mg/ml solution of PE were transferred to 4 conical plastic vials which were either empty (NONE), or contained 5 mg urea (UREA), 2 μl 50 mM dithiothreitol (DTT), or both urea and dithiothreitol (UREA + DTT). The vials were agitated gently for 10 min at 23° to dissolve the urea. To each vial was added 0.35 ml 50 mM tris, 5 mM EDTA, pH 8.1. Aliquots were diluted serially 10-fold in the same buffer and duplicate ADPR-transferase assays (5) were performed on 15- and 50-μl portions. The plateau at 7000 cpm results from consumption of the EF-2.

SDS gels regardless of whether the sample pretreatment included a reducing agent (5,9). In subsequent studies we have used tritium-labeled albumin as an internal mobility marker in SDS gels to show that treatment with reducing agent does not cause a measurable change in the molecular weight of the enzymatically active species. The method would have detected decreases in molecular weight of about 2,000. These studies show that the proenzyme does not contain a significant fraction of molecules with pre-existing peptide chain breaks ("nicks") between large peptide fragments. We also considered the possibility that activation results from release of a small inhibitor molecule or activation peptide. The requirement for simultaneous treatment by a denaturant and reductant could be explained if this presumptive inhibitor were buried within the protein.

To detect such a putative inhibitor we compared the amino acid content and terminal amino acids of native and activated PE. No significant difference was seen between the amino acid composition of native and activated samples which had been passed through Sephadex G-25 columns. A dimethylaminonaphthalene

sulfonyl chloride procedure, chosen to retain low molecular weight compounds, yielded alanine as the only N-terminal amino acid. [Our previous report (5) that the N-terminal amino acid is arginine was based on a single determination and was in error.] A further demonstration that amino acids or short peptides were not present was obtained by examining an acid hydrolysate of a PE sample which had been exhaustively methylated with [^{14}C]formaldehyde. The only radioactive compounds detected were N,N-dimethyl alanine and ε-N,N-dimethyl lysine, the latter being formed from the 15 residues of lysine per molecule (5). The ratio of radioactivity in these two compounds was consistent with a single N-terminal alanine per molecule. Additional evidence against release of a peptide was the coincidence of the single bands yielded by native and activated PE after isoelectric focusing in a polyacrylamide gel slab system capable of detecting single charge differences.

The results presented argue strongly that activation results from an unfolding and conformational rearrangement of the toxin molecule. However, there remains the possibility that the activation seen in Fig. 1 was an artifact due to unrecognized cleavage of toxin by proteases contained in the relatively crude wheat germ EF-2 extracts used in the ADPR-transferase assay. This possibility was excluded by showing that there was no lag in the time course of the ADPR-transferase reaction. Furthermore, identical results were obtained with highly purified rat liver EF-2 obtained from Elizabeth Maxwell, NIAMDD, Bethesda, Md.

A separate line of evidence that activation does not require proteolysis was obtained when it was found that PE, like DE, possesses latent NAD glycohydrolase activity. This activity, occurring when H_2O replaces EF-2 as substrate, occurs in DE at about 10^{-6} times the rate of ADPR-transfer to EF-2 (10). As shown in Table I, the activity of PE is increased at least 10-fold by simultaneous denaturation and reduction. Since the only protein present in NAD hydrolase assay mixtures is PE, which has no detectable protease (5), proteolytic activation cannot occur in this assay.

TABLE I. Exotoxin Catalyzed Hydrolysis of NAD

Toxin sample		NAD hydrolysis
Treatment	Amount assayed (μg)	(net cpm)
None	7	0
	70	80
Urea + DTT	8	280
	35	1390
	80	2270

The results cited demonstrate that the activation of PE resulting from denaturation and reduction is not the result of release of peptide fragments from a nicked molecule, nor is it a phenomenon resulting from unrecognized proteolytic fragmentation. Instead, it is most likely that activation results from a conformational rearrangment of the molecule that can occur only when internal hydrophobic interactions and disulfide bonds are simultaneously broken.

To obtain direct evidence that activation is correlated with disulfide bond rupture, these two processes were measured as a function of denaturant concentration (Fig. 2). PE samples were incubated in various concentrations of GuHCl containing 2 mM DTT and then divided into two portions. One of these was reacted with [^{14}C]iodoacetamide to measure sulfhydryls. The other portion was reacted with [^{12}C]iodoacetamide and assayed for ADPR-transferase activity. (Control studies had shown that blocking of all the cysteines of reduced PE does not decrease enzymatic activity.) The results show that (a) unfolding is a concerted event, (b) unfolding correlates with activation, and (c) the PE molecule is rather easily denatured (ribonuclease is typical of most proteins in its resistance to GuHCl denaturation). The valley in ADPR-transferase activity at 1.0 M GuHCl has been seen in three experiments, suggesting that intermediate concentrations of denaturant may allow disulfide bond interchange or trapping of partially denatured forms. The results of these experiments support the view that it is the unfolding of PE which causes activation. The

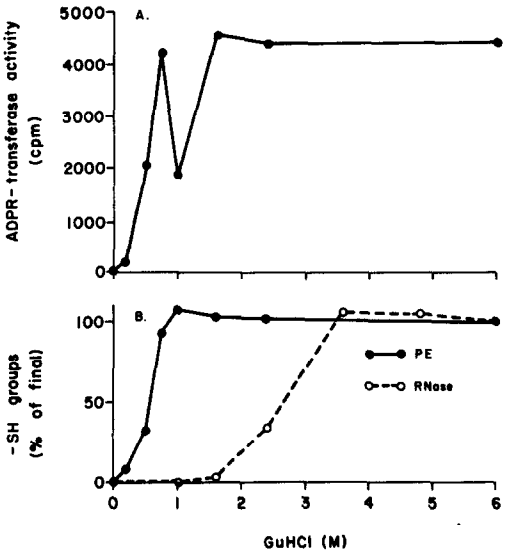

Figure 2. Activation of ADPR-transferase and reduction of disulfide bonds in PE as a function of guanidine hydrochloride (GuHCl) concentration. Samples of PE and ribonuclease (RNase) at 200 μg/ml were incubated for 90 min in 50 mM tris, 5 mM EDTA, 2 mM dithiothreitol (DTT), pH 8.5, containing the concentrations of GuHCl shown. All incubations were performed at 23°. (A) To measure ADPR-transferase activity, 20-μl aliquots of PE solutions were mixed with 2 μl of 50 mM iodoacetamide, incubated 15 min in the dark, diluted 1000-fold in 50 mM tris, 5 mM EDTA, 0.01% bovine albumin, pH 8.5, and assayed in duplicate on 10-μl portions. (B) To measure newly formed sulfhydryl groups, duplicate 20-μl aliquots of the PE and RNase solutions were mixed with 2 μl of 50 mM [1-^{14}C]iodoacetamide (approximately 1.8 mCi/mmol) incubated 15 min in the dark and transferred to paper discs which were processed to determine trichloroacetic acid-precipitable radioactivity (5).

apparent ease with which PE can be unfolded suggests that this process might occur under physiological conditions, perhaps with a hydrophobic region of the cell membrane acting as denaturant and glutathione present in the cytoplasm serving to break the disulfide bonds.

While activation by the unfolding mechanism described here is sufficient to explain the toxicity of PE, there is also evidence that an enzymatically active peptide fragment can be formed. In work described elsewhere (9,11) it has been shown that PE can be fragmented to yield an enzymatically active species approximately the size of fragment A of DE. Current evidence therefore suggests that PE can be activated in vitro by either of two procedures:

unfolding induced by reduction and denaturation as described here, or proteolytic fragmentation like that involved in activation of DE.

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