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REQUIREMENT OF THIOLS IN THE ADENOSINE-DIPHOSPHATE RIBOSYLATION OF ELONGATION FACTOR-2 BY *PSEUDOMONAS AERUGINOSA* EXOTOXIN A[1]

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Abstract. The extent of the transfer of the adenosine 5'-diphosphate ribose (ADPR) moiety of nicotinamide adenine dinucleotide onto elongation factor 2 (EF-2) catalyzed by *Pseudomonas aeruginosa* exotoxin A (PA-toxin) was dependent upon the presence of a reducing agent, dithiotheritol (DTT). The reaction requires DTT in low concentration (1 to 10 mM) and in the absence of DTT less product, ADPR-EF 2, was formed. PA-toxin was fully activated by treatment with a denaturing agent, sodium dodecyl sulphate (SDS), in conjunction with DTT. In the presence of activated toxin, the maximum transfer of ADPR onto EF-2 was observed when EF-2 had been previously reduced with DTT. Denaturation of EF-2 prior to reduction did not produce a further increase in its ability to act as a substrate for PA-toxin.

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Most strains of *Pseudomonas aeruginosa* produce a lethal exotoxin, PA-toxin, that may represent a major virulence factor of this organism (Bjorn *et al* 1977; Pavlovskis *et al* 1977). This toxin is a heat labile enzyme that can inhibit eucaryotic protein synthesis by catalyzing the transfer of the adenosine 5' diphosphate ribose (ADPR) moiety of nicotinamide adenine dinucleotide (NAD) onto elongation factor-2 (EF-2) by a reaction identical to that catalyzed by diphtheria toxin (Iglewski *et al* 1976, Iglewski *et al* 1977, Iglewski and Kabat 1975). In its native state, the toxin is an inactive proenzyme that can be activated by treatment with urea and dithiothreitol (DTT) (Leppla *et al* 1978) or by freezing at −20 °C and thawing (Vasil *et al* 1977). Activation by the latter method results in the production of an active fragment presumably due to the presence of a contaminating protease. Vasil's study reported that following activation, DTT was not required in the reaction. Our study presents evidence that a requirement exists for the presence of thiols in the ADP ribosylation of EF-2 by PA-toxin and that this requirement manifests itself as an increase in the extent of the reaction and is due to the reduction of EF-2.

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MATERIALS AND METHODS

Production and Purification of PA-Toxin

Pa-toxin was produced and purified from *P. aeruginosa* strain PA-103 as described previously (Callahan 1974, Liu *et al* 1973) with some modifications. The organisms were grown in the dialysate of Trypticase Soy Broth (BBL), supplemented with 1% glycerol and 0.05 M monosodium glutamate, for 18–22 hr at 32 °C with shaking.

The remaining steps were carried out at 5 °C. Bacteria were removed by centrifugation at 10,000 g for 40 min and the supernatant fluid of the culture was concentrated to 0.1 its original volume in an Amicon Model DC-2 hollow fiber dialyzer/concentrator fitted with a PM-10 Diaflo cartridge (Amicon). The concentrate was dialyzed 2 to 3 hr in the same unit against 0.01 M tris (hydroxymethyl) aminomethane-hydrochloride (Tris-HCl) (pH 8.0 at 25 °C), and another ten-fold concentration was achieved by precipitation with 80% saturated $(NH_4)_2SO_4$ and dissolution in 0.01 M Tris-HCl (pH 8.0 at 25 °C). After dialysis, this material was applied to a diethylaminoethyl (DEAE) cellulose column (24 x 1.5 cm) which had been equilibrated with 0.01 M Tris-HCl (pH 8.0 at 25 °C). Elution was achieved by a stepwise increase in ionic strength. Elution concentrations were 0.10 M, .23 M, and 1.0 M NaCl in 0.01 M Tris-HCl (pH 8.0 at 25 °C).

The 0.23 M NaCl eluate containing most of the ADP-ribosylation activity was concentrated to 5 ml by precipitation with ammonium sulphate as described above, and applied to a Sephadex G-200 column (50 x 2.5 cm) equilibrated with 0.01 M Tris-HCl (pH 8.0 at 25 °C). Fractions from the single peak of ADP-ribosylation activity were pooled and stored at −20 °C. SDS-polyacrylamide gel electrophoresis of 20 μg of the final product indicated a single band of protein with a M.W. of 70,000 daltons.

Assay for ADP-Ribosylation Activity

The enzymatic activity of the toxin was assayed as described by Collier and Kandel (1971). Extracts of rabbit reticulocytes containing EF-2 were prepared as described by Allen and Schweet (1962) except that DTT was eliminated from the preparation. EF-2 was quantified as described by Gill and Dinius (1973) except that PA-toxin was used in place of diphtheria toxin. The assay conditions are indicated below. A typical reaction contained 0.01 M Tris-HCl (pH 8.0 at 25 °C) 1 mM ethylenediaminetetra acetic acid (EDTA), 10 mM DTT, 3μM ^{14}C-NAD (140 μCi/μmole, Amersham), 0.8 nM EF-2, 0.5% BSA, and 10 μl of toxin containing the indicated concentration of protein in a final volume of 50 μl. Incubations were carried out for the indicated time periods at 25 °C and the reaction was stopped by the addition of 50 μl of 1 mM ^{12}C-NAD at 0 °C. The entire mix was spotted onto Whatman no. 3 MM scintillation pads (2.3 cm dia.) and processed as described by Bollum (1968). Protein was precipitated on the discs by 10% trichloroacetic acid (TCA), followed by two 15 min washes in 5% TCA and two 10 min washes in 100% ETOH. The discs were dried and counted in 5 ml of NEN-949 (New England Nuclear) for 5 min in a Beckman LS-233 liquid scintillation counter. Counting efficiency was consistantly above 85%.

Effect of DTT on Toxin Activity

In order to examine the effect of DTT on the activity of PA-toxin, toxin at 200 μg/ml was incubated in the presence of 2 mM EDTA, 1% BSA, and increasing concentrations of DTT for 60 min at 25 °C in a volume of 25 μl. EF-2, ^{14}C-NAD and sufficient buffer to bring the volume to 50 μl were added and incubated for 15 min at 25 °C. The assay was terminated and the discs washed and counted as described above.

Kinetics of the activity of PA-toxin exposed to DTT were examined by incubating the toxin with 10 mM DTT for 10 min, adding the remaining components of the reaction mix and stopping the reaction at various times. A duplicate set of timed samples obtained from toxin not exposed to DTT was used for comparison.

Denaturation and Reduction of EF-2 and PA-toxin

The toxin and EF-2 preparations were treated as described by Konigsberg (1972) with modifications. PA-toxin at 3 μg/ml and the crude EF-2 extract containing 4 pmoles/ml of EF-2 were denatured with 0.1% sodium dodecyl sulphate (SDS) in 0.01 M Tris-HCl (pH 8.0 at 25 °C) 1 mM EDTA for 1 hr at 25 ° C. Following this treatment, DTT was added to a final concentration of 10 mM and incubated for 2.5 hr at 25 °C. Finally, N-ethylmaleimide (NEM) was added to a final concentration of 40 mM and the incubation was continued for 30 min at 25 °C. Samples were dialyzed overnight at 5 °C against 0.01 M Tris-HCl (pH 8.0 at 25 °C). NEM alkylates the free sulfhydryl groups, preventing the reforming of the disulfide bridges normally occurring following removal of the DTT by dialysis.

We examined the effect of reduction without denaturation by treatment of the toxin or EF-2 with 10 mM DTT followed by alkylation with 40 mM NEM as above. The effect of denaturation alone with 0.1% SDS and the effect of alyklation of free sulfhydryls with 40 mM NEM alone was also studied. Controls consisted of samples of toxin or EF-2 not receiving any treatment. All samples were dialyzed overnight at 5 °C against 0.01 M Tris-HCl (pH 8.0 at 25 °C). Toxin and EF-2 were assayed as described above except that DTT was omitted from the reaction mixtures.

Data for the effects of denaturation and reduction of PA-toxin and EF-2 were analyzed by the two-way analysis of variance and transformed to common logarithms prior to analysis. Differences between means were analyzed by the Student-Newman-Kuels test.

RESULTS AND DISCUSSION

Contrary to the results of other investigators (Vasil *et al* 1977), we have consistently noted an effect of DTT alone on the ADP-ribosylation of EF-2 by PA-toxin. The effect of DTT was dependent upon its concentration in the reaction mixture although the activity reached a plateau at 1 mM DDT (figure 1).

A comparison of the kinetics of the reaction in the presence of DTT with the kinetics in the absence of DTT demonstrated that the initial rates of the reactions were the same but the extent of the reaction was greater in the presence of DTT (figure 2). These results suggested that the DTT was acting on the substrate, EF-2, since more of final product was being formed.

Examination of the effects of exposure of EF-2 to DTT followed by alkylation with NEM and extensive dialysis to remove the free DTT substantiated this hypothesis (table 1). Untreated toxin assayed in the presence of reduced and alkylated EF-2 showed a significantly higher transfer of radioactivity into acid precipitable material ($P<0.01$). Exposure of EF-2 to a denaturant, SDS,

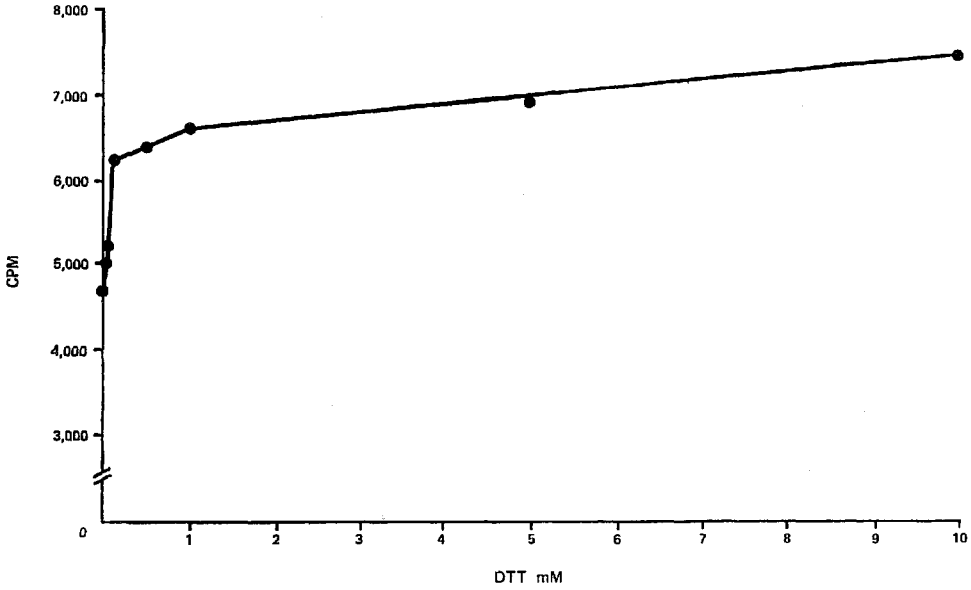

FIGURE 1. Effect of dithiothreital (DTT) concentration on the ADP-ribosylation activity of PA-toxin. Each point is the average of duplicate measurements.

prior to reduction with DTT did not increase the extent of the reaction further than treatment with DTT alone. Exposure to 40 mM NEM or 0.1% SDS had no significant effect on the ability of EF–2 to act as a substrate for PA-toxin.

The results obtained following exposure of PA-toxin to SDS and DTT are consistent with those of Leppla *et al* (1978) and Vasil *et al* (1977). Activation of PA-toxin was observed only following denaturation and reduction.

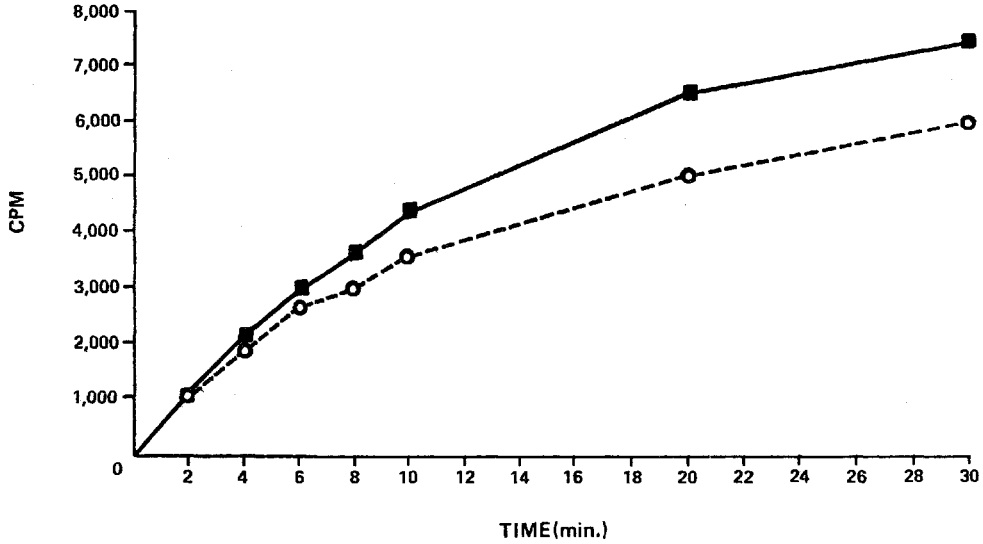

FIGURE 2. ADP-ribosylation activity of PA-toxin in the presence (■———■) and absence (○- - -○) of 10 mM DTT. A typical reaction mix contained 0.01 M Tris-HCl (pH 8.0), 1 mM EDTA, 3 μm ^{14}C-NAD (140 μCi/umole), 0.8 uM EF-2, 0.5% BSA, and 100 μg/ml PA-toxin in a volume of 50 μl. Each point is the average of duplicate measurements.

TABLE 1. *Treatment of PA-toxin and EF-2 prior to assay for ADP-ribosylation activity.**

Treatment of PA-toxin (3 μg/ml)	Treatment of EF-2 (4 nmoles/ml)				
	None	NEM**	SDS+	DTT++	SDS & DTT†
None	716††	893	835	1810	1660
NEM	656	832	836	1670	1640
SDS	598	795	756	1690	1570
DTT	777	911	955	2100	1840
SDS & DTT	1280	1280	1340	2770	2330

*A 10 μl sample of toxin and EF-2 were assayed as described.
**40 nM N-ethyl malamide 30 min.
+0.1% sodium dodecyl sulphate 1 hr.
++10 mM dithiothreital (DTT) 2.5 hr; then 40 mM NEM, 30 min.
†0.1% SDS, 1 hr; then 10 mM DTT, 2.5 hr; then 40 mM NEM, 30 min.
††Average CPM of duplicate measurements.

We conclude that in order for EF–2 to accept the ADPR moiety of NAD in the reaction catalyzed by PA-toxin, the disulfide bonds of EF–2 must be reduced. The sulfhydryls are not, however, the site of attachment of ADPR since blocking with NEM does not inhibit the reaction. In the internal environment of the cell, the EF–2 probably exists in the reduced state but during extraction some disulfide bonds form. It is important that investigators studying the activation of PA-toxin, and possibly other enzymes that require thiols for activity, take into consideration the effects of these agents on the substrates.

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