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Pseudomonas aeruginosa Exotoxin A: Alterations of Biological and Biochemical Properties Resulting from Mutation of Glutamic Acid 553 to Aspartic Acid†

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Received November 14, 1989; Revised Manuscript Received January 19, 1990

ABSTRACT: Glutamic acid 553 of *Pseudomonas aeruginosa* exotoxin A (ETA) was identified earlier as a putative active-site residue by photoaffinity labeling with NAD. Here ETA-E553D, a cloned form of the toxin in which Glu-553 has been replaced by aspartic acid, was purified from *Escherichia coli* extracts and characterized. Cytotoxicity of the mutant toxin for mouse L-M cells was less than 1/400 000 that of the wild type. The mutation caused a 3200-fold reduction in NAD:elongation factor 2 ADP-ribosyltransferase activity, as estimated by assays with an active fragment derived from the toxin by digestion with thermolysin. NAD glycohydrolase activity was reduced somewhat less, by a factor of 50, and photoaffinity labeling with NAD by a factor of 2. We detected less than 2-fold change in the values of K_M for NAD or elongation factor 2 and no change in K_D for NAD, as determined by quenching of protein fluorescence. The drastic reduction of ADP-ribosyltransferase activity therefore results primarily from an effect of the mutation on k_{cat}, implying that Glu-553 plays an important and possibly direct role in catalyzing this reaction. The effects of the E553D mutation are similar to those of the E148D mutation in diphtheria toxin, supporting the notion that these two Glu residues perform the same function in their respective toxins.

Exotoxin A of *Pseudomonas aeruginosa* (ETA;[1] 613 residues, M_r 66 583) belongs to a class of bacterial toxins that ADP-ribosylate target proteins of eucaryotic cells (Jacobson & Jacobson, 1989; Collier & Mekalanos, 1980). Like diphtheria toxin (DT), ETA is a proenzyme, which, after activation, catalyzes transfer of the ADP-ribose moiety of NAD to the diphthamide residue of elongation factor 2 (EF-2). This blocks the translocation step of protein synthesis, thereby causing cell death.

Photoaffinity labeling and directed mutagenesis have been used to identify a putative active-site residue of ETA. Carroll and Collier (1987) found that radiolabel from nicotinamide-labeled NAD was efficiently transferred to a single site, position 553, of an enzymically active thermolysin fragment (A_{tl}) of the toxin when the mixture was irradiated with 254-nm ultraviolet light (Carroll & Collier, 1987). The photoproduct at position 553 was chromatographically identical with that formed at position 148 in diphtheria toxin (DT) fragment A irradiated under similar conditions. In the DT fragment Glu-148 was modified to give a photoproduct containing the nicotinamide ring of NAD linked by its carbon 6 to the decarboxylated γ-methylene group (Carroll et al., 1985). The structure of the protoproduct suggests that the carboxyl function may be in contact with the *N*-glycosyl linkage ruptured during ADP-ribosyl transfer and hence directly involved in catalysis.

To probe the role of Glu-553 of ETA in ADP-ribosylation activity, NAD glycohydrolase activity, cytotoxicity, and photolabeling with NAD, we substituted Asp for this residue in cloned ETA and expressed the mutant gene in *Escherichia coli* (Douglas & Collier, 1987). Earlier, ETA was expressed in *E. coli* under control of the hybrid *tac* promoter, and it was found that the gene product, in crude extracts, was indistinguishable from ETA produced by *P. aeruginosa* with regard to toxicity and enzymic activity (Douglas et al., 1987). When the Glu-553 → Asp mutant toxin, ETA-E553D, was expressed in *E. coli* and assayed in crude extracts, its ADP-ribosylation and cytotoxic activities were found to have been reduced by greater than 1800-fold and 10 000-fold, respectively (Douglas & Collier, 1987). Here we report purification and more detailed characterization of ETA-E553D obtained by expression of the mutant cloned gene in *E. coli*.

Experimental Procedures

Bacterial Strains. E. coli JM103 (pCDPT2) and *E. coli* JM103 (pCDPTE553D) were described recently (Douglas et al., 1987; Douglas & Collier, 1987).

Purification of ETA and ETA-E553D from E. coli. Exotoxin A produced by *P. aeruginosa* PA103 was purified as described (Lory & Collier, 1980) and used as a reference throughout these studies. Overnight cultures of *E. coli* JM103 (pCDPT2) and JM103 (pCDPTE553D) in L-broth with 100

† This work was supported by USPHS Grants AI22021 and AI22848 from the National Institute of Allergy and Infectious Diseases.

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[1] Abbreviations: ETA, *Pseudomonas aeruginosa* exotoxin A; DT, diphtheria toxin; A_{tl}, enzymically active fragment of ETA generated by digestion with thermolysin.

0006-2960/90/0429-5043$02.50/0

μg/mL ampicillin were diluted to an initial A_{595} of 0.1 in 2 L of the same medium supplemented with Antifoam B (Baker Chemical Co.). Cultures grown at 37 °C in Kleuver-like flasks were sparged with oxygen at a flow rate of 1.5 L/min. Isopropyl β-D-thiogalactopyranoside from Sigma was added after 1.5 h to a final concentration of 1 mM. Six hours later, cells were sedimented by centrifugation at 4000*g* for 15 min at 4 °C, washed with 10 mM Tris-HCl (pH 8.0), and resuspended in 20 mL of 5 mM Tris-HCl (pH 8.2) and 0.1 mM EDTA (TE). We sonicated cell suspensions (Branson Sonic Power Co.) and removed insoluble material by centrifugation at 100000*g* at 4 °C for 20 min. Solid ammonium sulfate (Schwarz/Mann Biotech) was added to a final concentration of 1 M; after 30 min of gentle agitation, precipitated material was sedimented at 30000*g* for 30 min at 4 °C. The supernatant fraction was then subjected to sequential steps of chromatography on phenyl-Sepharose, DEAE-Sephacel, and an immunoaffinity resin, as described below.

Column fractions were assayed for immunoreactivity, absorbance at 280 nm, and ionic strength. To visualize immunoreactive proteins, aliquots (5–10 μL) were applied to nitrocellulose sheets presoaked in 192 mM glycine, 25 mM Tris-HCl (pH 8.3), and 0.1% SDS and inserted into a slot blotter transfer apparatus (Schleicher & Schuell). The filters were vacuum-aspirated for 30 s and immersed in blocking buffer [10 mM Tris-HCl (pH 8.0), 2 mM EDTA, 50 mM NaCl, and 0.1% hemoglobin] for 30 min with gentle agitation. Blots were probed by the method of Tweten and Collier (1983), using 150 μg of rabbit anti-ETA IgG (Douglas et al., 1987) and 1×10^7 cpm of protein A (Sigma Chemicals) labeled with ^{125}I (Amersham Corp.; 14.6 mCi/mg) to a specific activity of 8.3×10^7 cpm/mg (Hunter & Greenwood, 1962). Estimates of the amount of immunoreactive protein were interpolated from the intensity of autoradiographic bands (LKB Ultroscan) produced by known amounts of ETA. Absorbance at 280 nm was measured with a Uvikon 820 spectrophotometer (Kontron Instruments), and the salt concentration of column effluent was estimated with a conductivity monitor (Bio-Rad Laboratories). Protein concentrations were estimated by the method of Bradford (1976). SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of proteins in column fractions was performed as described by Laemmli (1970). Samples were electrophoresed on 11.25% gels (Hoeffer Scientific) and proteins were visualized by staining with 0.01% Coomassie blue.

The supernatant fraction from the ammonium sulfate precipitation was applied at a flow rate of 17 mL/h to a 2.5-cm by 16.5-cm column of phenyl-Sepharose (Sigma Chemicals) equilibrated in 1 M $(NH_4)_2SO_4$ in TE. After washing the column with equilibration buffer to remove unbound material, we applied a gradient of decreasing ammonium sulfate (1.0 → 0.2 M). ETA-related material was then eluted with 2% ethylene glycol in TE; fractions were assayed as described for material that reacted with anti-ETA IgG and comigrated with ETA by SDS–PAGE. The wild-type protein consistently eluted slightly earlier than the mutant. Peak fractions were combined and dialyzed against several changes of TE at 4 °C.

Pooled material from phenyl-Sepharose chromatography was applied at 18 mL/h to a 1.5-cm by 16-cm DEAE-Sephacel column (Pharmacia) preequilibrated with TE. After the sample was loaded, the column was washed with >10 column volumes of TE before applying a gradient of NaCl in TE (0–0.5 M). Fractions were assayed for cross-reacting material (CRM) by immunoblotting and for protein by SDS–PAGE. Both wild-type and mutant proteins eluted at about 0.25 M NaCl. We combined the peak fractions and brought the volume to 20 mL with TE containing 150 mM NaCl (TES).

Immunoaffinity purification of ETA and ETA-E553D from DEAE pools was performed by a modification of the procedure of Taylor and Pollack (1978). An IgG fraction from ETA-immunized rabbit serum was prepared as described (Douglas et al., 1987), and antibodies were coupled to Affi-Gel 10 (Bio-Rad Laboratories) as recommended by the manufacturer. Briefly, 100 mg of IgG was coupled to 20 mL of washed resin in 0.1 M MOPS (pH 7.0) for 12 h at 4 °C. Unreacted active ester groups were subsequently blocked with a 1-h incubation with 2 mL of 1 M ethanolamine hydrochloride (pH 8.0); over 90% of the available protein was coupled. The anti-ETA immunoaffinity resin was washed with TES and packed into 10-mL Pharmaseal syringes (American Scientific Products), with final column dimensions of 1.5 cm × 3.5 cm. Pooled immunoreactive material from each DEAE column was circulated through the affinity columns at 10 cm/h for 2.5 h. Unbound protein was washed from the column with 5 volumes of TES, and bound protein was eluted with 3 M KCSN in TES followed by dialysis against 50 mM Tris-HCl (pH 8.2) and 1 mM EDTA (TE10). ETA-E553D and ETA were concentrated with Centriprep 30 microconcentrators (Amicon, Inc.) to a final volume of 0.6 mL and stored frozen at −20 °C.

Activation and Digestion with Thermolysin. Purified ETA-E553D and ETA were activated in the presence of 6 M urea and 10 mM DTT and carboxymethylated by the method of Carroll and Collier (1987). The activated toxins were desalted on 0.8-cm × 25-cm columns of Sephadex G-50 F (Pharmacia Fine Chemicals) in 50 mM Tris-HCl (pH 8.0) at a flow rate of 25 cm/h, and the peak of material that absorbed at 280 nm was stored frozen in aliquots at −20 °C. Analytical thermolysin digestions were performed with 3 μM activated toxin in the presence of 2 mM NAD. Thermolysin (Calbiochem) was added to digestion mixtures at final concentrations of 100 nM, 200 nM, and 1 μM; after 30 min at 25 °C, EDTA was added to 5 mM, and the digestion mixtures were boiled in the presence of SDS and β-mercaptoethanol. Proteins were separated on 12.5% gels, stained with Coomassie blue, and destained thoroughly as described (Laemmli, 1970). Relative amounts of ETA A_{t1} fragments from wild-type and mutant ETA were quantified with an Ultroscan XL laser densitometer (LKB Instruments).

To prepare thermolytic fragments for enzymic and spectral characterization, activated wild-type or mutant ETA was diluted to a final concentration of 3 μM and digested with 100 nM thermolysin in the presence of 2 mM NAD. Products of the digestion were separated on a Superose 12 gel filtration column using the FPLC system of Pharmacia, Inc. The column was equilibrated and eluted with 25 mM Tris-HCl (pH 8.2), 1 mM EDTA, and 150 mM NaCl at a flow rate of 0.3 mL/min. Absorbance at 254 nm was monitored (Pharmacia, Inc.), peak fractions were analyzed by SDS–PAGE, and those containing material that comigrated with the A_{t1} fragment were pooled, concentrated in Centricon 10 microconcentrators (Amicon, Inc.), and frozen in aliquots at −20 °C. The possibility for cross-contamination between wild-type and mutant A_{t1} fragments was minimized by flushing the system with 0.1 N NaOH and 2 M NaCl before and after each digestion mixture was applied to the column.

Enzymic Assays. ADP-ribosyltransferase assays were performed as described (Douglas & Collier, 1987). Samples containing whole toxins were activated with 4 M urea and 10 mM DTT for 30 min at 25 °C before being diluted 10-fold into reaction mixtures containing 0.5 μM EF-2 and 2 μCi of

Table I: Purification of Wild-Type and E553D ETA from *E. coli*

purification step	total protein (mg)	immuno-reactivity	enzymic activity	cytotoxic activity	specific immunoreactivity (units/mg of protein)	purification factor
			(A) Wild-Type ETA			
crude extract	462 ± 6	2.9 ± 0.5	3.0	3.1	0.01	(1)
$(NH_4)_2SO_4$ supernatant	266 ± 7	2.2 ± 0.3	2.3	1.7	0.01	1.3
phenyl-Sepharose	138 ± 4	1.6 ± 0.1	1.9	1.5	0.01	1.8
DEAE-Sephacel	47 ± 2	1.6 ± 0.1	1.5	1.5	0.03	5.6
immunoaffinity	0.35 ± 0.04	0.35 ± 0.05	0.45	0.40	0.99	165
			(B) ETA-E553D			
crude extract	476 ± 6	2.5 ± 0.1	4.3×10^{-4}	$<3.8 \times 10^{-5}$	0.01	(1)
$(NH_4)_2SO_4$ supernatant	293 ± 7	1.8 ± 0.1	4.0×10^{-4}	$<3.1 \times 10^{-5}$	0.01	1.2
phenyl-Sepharose	133 ± 4	1.4 ± 0.1	4.1×10^{-4}	$<1.1 \times 10^{-5}$	0.01	2.2
DEAE-Sephacel	44 ± 2	1.4 ± 0.1	3.1×10^{-4}	$<1.7 \times 10^{-5}$	0.03	6.4
immunoaffinity	0.37 ± 0.04	0.38 ± 0.05	9.0×10^{-5}	$<0.8 \times 10^{-6}$	1.03	206

[*adenylate*-^{32}P]NAD (991 Ci/nmol; Amersham Corp.) in TE10. After 1 h, protein was precipitated with TCA and assayed for incorporation of label. Carboxymethylated toxins or the active thermolytic fragments were not treated with urea and DTT prior to assay.

The NAD glycohydrolase activity of purified toxins and proteolytic fragments was determined by a modification of the method of Kandel et al. (1974). Mixtures containing activated whole toxins or thermolytic peptides and 3.2 μM [4-^{3}H]NAD (3.2 Ci/mmol; Amersham Corp.) were incubated for 13 h at 37 °C. The reaction was terminated by the addition of $Na_2B_4O_7$ or sodium borate to 150 mM, and radiolabeled nicotinamide was extracted with water-saturated ethyl acetate for liquid scintillation counting.

Kinetics of ADP-Ribosylation. Mutant A_{tl} fragment at 23 nM and wild-type fragment at 5 pM were assayed for ADP-ribosyltransferase activity at various substrate concentrations (Allison & Purich, 1979). The specific activity of [*adenylate*-^{32}P]NAD was diluted from 990 to 2 Ci/nmol with unlabeled NAD (Boehringer-Mannheim). Final concentrations of NAD in reaction mixtures varied from 5.6 to 50 μM; EF-2 was diluted such that the concentration varied from 0.11 to 1.0 μM. Nonspecific incorporation of radiolabel into TCA-precipitable material was subtracted for each set of substrate conditions. Incubations carried out for 30 min at 25 °C were performed in duplicate.

Photoaffinity Labeling. We photoaffinity-labeled ETA and the thermolysin fragments (A_{tl}) from ETA and ETA-E553D as described by Carroll and Collier (1987). Briefly, samples containing 2 μM protein and 40 μM [4-^{3}H]NAD (2.0 Ci/mmol; Amersham Corp.) in TE10 were applied as droplets to plastic dishes at 0 °C. Protein–ligand mixtures were irradiated with a GE germicidal lamp (254 nm) at an intensity of 2.5 mW/cm^2. Aliquots were removed from the drops at specified times, added to 20 volumes of 500 μg/mL bovine serum albumin in TE10, and mixed with ice-cold 10% TCA. Precipitates were sedimented by centrifugation, washed twice with TCA, dried, and dissolved in 0.1 N NaOH before liquid scintillation counting.

Cytotoxicity. Cytotoxic activity against mouse L-M fibroblasts was determined as described by Carroll and Collier (1987).

Results

Purification and Partial Characterization of E553D and Wild-Type ETA. E553D and wild-type ETA were purified from *E. coli* clones bearing plasmids pCDPTE553D and pCDPT2, respectively, and compared with authentic ETA from *P. aeruginosa* (Table I). Extracts obtained by sonication of *E. coli* were fractionated sequentially by hydrophobic interaction, ion exchange, and immunoaffinity chromatographic steps. After each step of the purification protocol we measured specific immunoreactivity, cytotoxicity, and ADP-ribosyltransferase activity and monitored the band patterns formed on SDS–polyacrylamide gels (Table I, Figure 1) and Western blots. Immunoreactivity was assayed by radioimmunoassay, and cytotoxicity was determined by measuring protein synthesis in mouse L-M cells after a 5-h incubation with varying dilutions of toxin. To assay ADP-ribosyltransferase activity of the whole toxin, the native protein, a proenzyme, was first treated with dithiothreitol in the presence of 4 M urea to convert it to an enzymically active form. ADP-ribosyltransferase assay mixtures contained ca. 20 nM [*adenylate*-^{32}P]NAD ($\ll K_D$) and 0.5 μM EF-2 ($\sim K_M$).

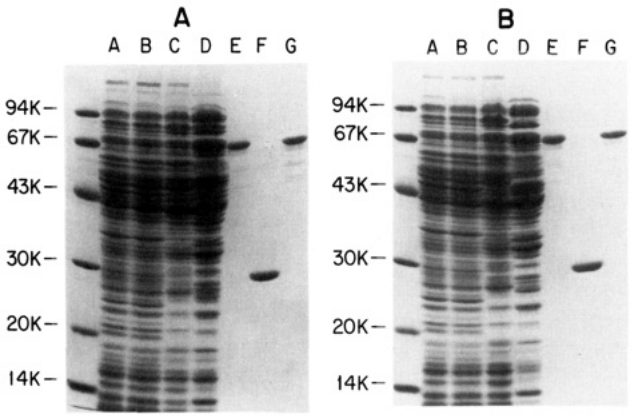

FIGURE 1: SDS–polyacrylamide gel electrophoresis of samples of ETA and ETA-E553D from various steps of purification. Electrophoresis was on an 11.25% gel, and staining was with Coomassie blue. Quantities of protein per lane are given in parentheses below. Panel A, ETA; panel B, ETA-E553D. Lane A, dialyzed high-speed supernatant fraction from sonicated cells (50 μg); lane B, $(NH_4)_2SO_4$ supernatant fluid (50 μg); lane C, pooled material from phenyl-Sepharose column (50 μg); lane D, pooled material from DEAE-Sephacel column (50 μg); lane E, pooled material from immunoaffinity column (1.8 μg); lane F, A_{tl} fragment (2 μg); lane G, ETA standard (2 μg).

The chromatographic profiles for the mutant and wild-type forms of ETA were nearly identical at each stage of the purification (data not shown). Both proteins were enriched slightly, and to the same extent, after chromatography on phenyl-Sepharose and DEAE-Sephacel (Table I). Although the purification obtained with these resins was not extensive, the similarity of the toxin elution profiles demonstrated that the Glu-553 → Asp mutation did not significantly alter the chromatographic properties of the protein. These chromatographic steps reduced the total protein applied to the immunoaffinity resin in the final step, which provided most of the purification.

Relative to immunoreactivity, the enzymic and cytotoxic activities of the wild-type and mutant proteins remained constant throughout the purification, and the activities of the wild-type toxin purified from *E. coli* were identical with those of the standard toxin from *P. aeruginosa* PA103. These findings and the fact that the 67-kD full-length toxin band was the only one seen on Western blots at any step in the purification of both the mutant and wild-type proteins implied that the E553D mutation was not accompanied by significant loss of stability.

On SDS–polyacrylamide gels, purified ETA-E553D comigrated with ETA from *E. coli* or from *P. aeruginosa* (Figure 1). The purity of the final products was greater than 95%, as estimated by laser densitometry of Coomassie blue stained gels. The near-UV absorption spectra and fluorescence emission spectra of the purified proteins were identical, as were spectra of the A_{tl} fragments (data not shown).

Purified E553D toxin exhibited drastically reduced cytotoxicity and ADP-ribosyltransferase activity but had the same specific immunoreactivity as the wild-type protein (Table I). The low, but readily measurable, ADP-ribosyltransferase activity was approximately 1/3200 that of ETA. This residual activity was specific for EF-2 as demonstrated by electrophoresis of the reaction mixture on SDS–polyacrylamide gels, followed by autoradiography. Cytotoxic activity, on the other hand, was undetectable at any step of purification. From the highest concentration of purified ETA-E553D tested, we concluded that its cytotoxicity was diminished by a factor of at least 400000. Purified wild-type ETA from *E. coli* showed no significant difference from ETA produced by *P. aeruginosa* with respect to any of the activities assayed.

Replacement of the *Xho*I–*Hin*dIII restriction fragment of the mutant toxin gene with the corresponding wild-type fragment yielded ETA with wild-type levels of cytotoxicity, and sequencing of the mutant *Xho*I–*Hin*dIII fragment confirmed that it contained only a single mutated codon, specifying the E553D mutation (Douglas & Collier, 1987). Thus it is unlikely that there was a second mutation, lying outside the *Xho*I–*Hin*dIII fragment, that contributed to the drastic reduction in cytotoxicity.

Preparation of the A_{tl} Fragment. For more detailed measurements of enzymic properties, an enzymically active fragment, ETA-A_{tl}, was prepared (Carroll & Collier, 1987). Unlike activated whole toxin, ETA-A_{tl} was stable and active in the absence of urea or reducing agents. Thus complications in analytical measurements due to the presence of urea were avoided, and the degree of reduction of the toxin was eliminated as a potential variable. ETA-A_{tl} was generated by thermolysin digestion of purified ETA that had first been activated in urea/dithiothreitol and carboxymethylated. NAD was included in the digestion mixture to protect the enzymic domain against proteolytic attack. The active fragment was then separated from other digestion products and NAD by chromatography on a Superose 12 FPLC column.

Wild-type A_{tl} fragment showed 3–5-fold higher activity than activated whole ETA in the ADP-ribosylation and NAD glycohydrolase assays (Table II). The thermolysin fragment from ETA-E553D was ca. 1/3000 as active as the wild-type peptide in the ADP-ribosyltransferase assay. NAD glycohydrolase activity was also reduced by the E553D mutation, but only about 50-fold.

Kinetic Measurements. Kinetic measurements of NAD:EF-2 ADP-ribosyltransferase activity were made to explore the nature of the catalytic defect resulting from the E553D mutation. The purified A_{tl} fragment from ETA or ETA-E553D (5 pM or 23 nM, respectively) was assayed for ADP-ribosyltransferase activity in the presence of concentrations of [*adenylate*-^{32}P]NAD and EF-2 spanning the expected values of K_M. Conditions were chosen such that less than 10% of either substrate was consumed during the 30-min reaction period. NAD concentrations were varied from 5.6 to 50 μM and EF-2 concentrations from 0.11 to 1.0 μM. Eadie–Hofstee plots of the data yielded a K_M value for EF-2 of 0.36 ± 0.1 μM for the wild-type fragment and 0.47 ± 0.1 μM for the mutant fragment (Figure 2). The Michaelis constant measured for NAD was 35 ± 12 μM for the wild-type fragment and 59 ± 20 μM for the mutant (data not shown). The differences between wild-type and mutant fragments are therefore slight, relative to the overall differences in enzymic activity.

Table II: Enzymic Activities of Purified Wild-Type and E553D ETA[a]

protein	ADP-ribosylation		NAD glycohydrolase activity	
	whole toxin	A_{tl} fragment	whole toxin	A_{tl} fragment
wild type (*P. aeruginosa*)	17	45	0.016	0.093
wild type (*E. coli*)	16	42	0.016	0.061
E553D mutant	0.003	0.013	ND[b]	0.0012

[a] ADP-ribosyltransferase activity was assayed with [*adenylate*-^{32}P]-NAD and partially purified EF-2, as described under Experimental Procedures. Units are moles of ADP-ribosylated EF-2 formed per hour per mole of enzyme. NAD glycohydrolase activity is expressed as moles of NAD hydrolyzed per hour per mole of enzyme, as described. Values are the average of assays performed in duplicate, at no less than three concentrations of enzyme. Whole toxin molecules had been activated, carboxymethylated, and desalted. [b] Not determined.

The affinity of NAD for the A_{tl} fragments derived from wild-type toxin, ETA-E553D, or ETA from *P. aeruginosa* was also determined directly by measuring the change in intrinsic protein fluorescence as a function of NAD concentration (Figure 3). Identical values, 36 ± 8 μM, were found in each case, indicating that the E553D mutation did not significantly affect affinity for NAD.

The E553D mutation caused no significant effect on K_M for either NAD or EF-2 or the K_D for NAD, thus implying an alteration of k_{cat}. We estimated that the k_{cat} for the ADP-ribosylation of EF-2 catalyzed by the E553D active fragment was about 1/4000 that of the wild-type fragment.

Photoaffinity Labeling. The thermolysin fragment of ETA-E553D was subjected to UV radiation in the presence of [4-^{3}H]NAD to determine the effect of the mutation on photolabeling of the residue at position 553. As shown in Figure 4, the initial rate of photolabeling and the maximal level of incorporation of label were about half those of the wild-type fragment. Native ETA was also photolabeled to a relatively low level, but we observed, reproducibly, a lag period of several minutes before labeling could be detected. Native toxin is known to bind NAD only weakly, and we believe that photochemical damage to other parts of the toxin, such as the disulfide bridges, may relieve conformational constraints on the enzymic domain, permitting it to bind NAD more tightly and become photolytically competent. No attempt was made in these experiments to confirm the site of photolabeling or the nature of the photoproduct.

Thermolysin Sensitivity. To probe the effects of the E553D mutation on conformation of the enzymic domain, we examined the differential sensitivity of ETA and ETA-E553D to digestion with thermolysin. Activated, carboxymethylated mutant and wild-type toxins were digested with a range of

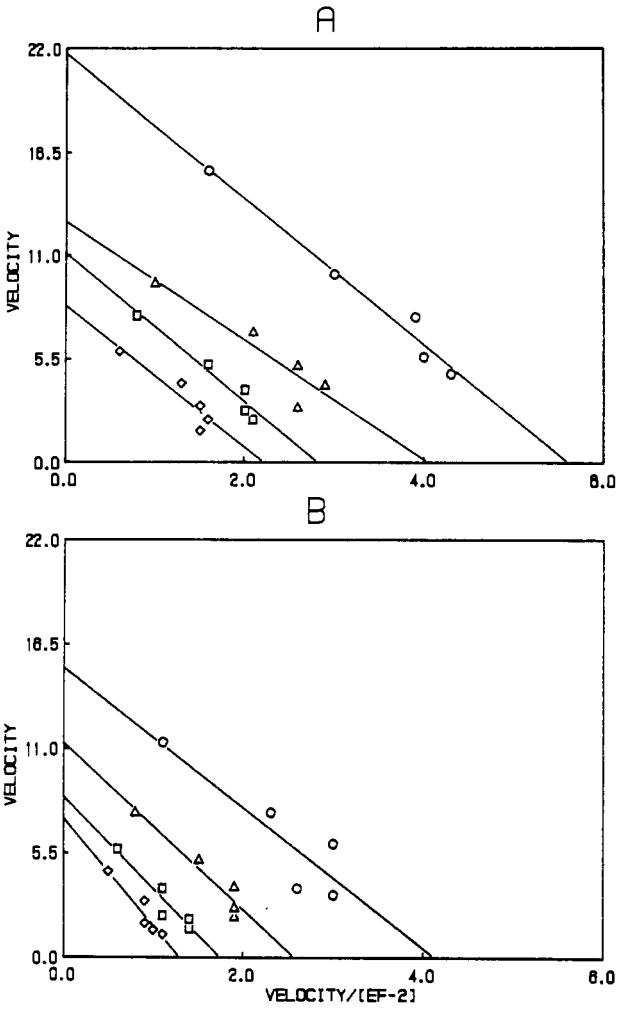

FIGURE 2: Eadie–Hofstee plots of initial velocities of ADP-ribosylation as a function of EF-2 concentration. Enzyme was wild-type A_{tl} (panel A) or E553D A_{tl} (panel B). EF-2 concentrations ranged from 0.11 to 1 μM. Units on the abscissa are pmol of ADP-ribosylated EF-2/h, and units on the ordinate are L/h $\times$ 10^7. Concentrations of NAD were as follows: ○, 17 μM; △, 10 μM; □, 7.1 μM; ◇, 5.6 μM.

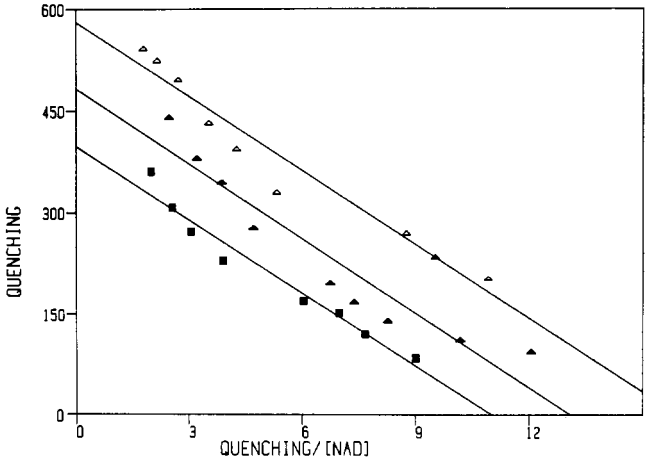

FIGURE 3: Eadie–Hofstee plot of quenching of A_{tl} fragment fluorescence by NAD. A_{tl} fragments were derived from the following sources: ETA from *P. aeruginosa*, △; ETA from *E. coli*, ▲; and ETA-E553D, ■. The lines were determined by linear regression analysis of the data; the slope of each was 36 μM.

thermolysin concentrations in the presence of NAD. Each digestion was terminated by addition of EDTA, the protein products were fractionated by SDS–PAGE, and the relative amounts of A_{tl} produced with different concentrations of thermolysin were quantified by laser densitometry. No undigested whole toxin was found in any of the samples, indicating complete conversion to smaller fragments. Whereas the yield of wild-type A_{tl} increased as the concentration of thermolysin was increased from 5 to 50 μg/mL, the yield of A_{tl} from ETA-E553D decreased over the same range of thermolysin concentrations (Figure 5). In the absence of NAD, both wild-type and mutant forms of ETA were digested to peptides that were not detectable on the polyacrylamide gels.

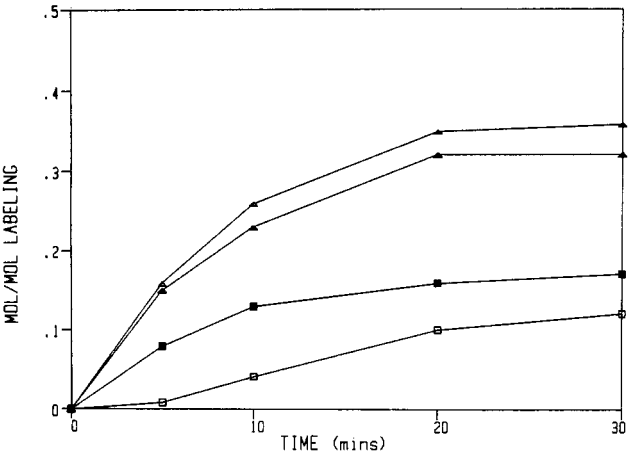

FIGURE 4: Photoaffinity labeling of mutant and wild-type A_{tl} fragments. The incorporation of label from [4-^{3}H]NAD into the following fragments is shown: ETA, □; A_{tl} from ETA (*E. coli*), ▲; A_{tl} from ETA (*P. aeruginosa*), △; A_{tl} from ETA-E553D, ■. Ovalbumin was irradiated under the same conditions as a control, and incorporation of label at each time point was subtracted from the samples as nonspecific incorporation.

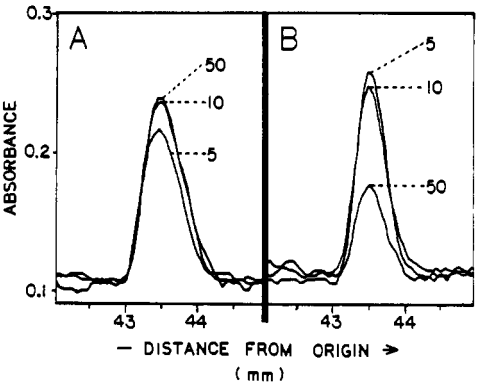

FIGURE 5: Yields of A_{tl} fragment from digestion of activated, carboxymethylated wild-type, or mutant ETA with various concentrations of thermolysin. ETA (*E. coli*) or ETA-E553D was digested with 5, 10, or 50 μg of thermolysin/mL, as indicated in each panel, in the presence of 2 mM NAD at 25 °C. Reactions were terminated after 30 min by addition of EDTA to 10 mM. SDS sample buffer with β-mercaptoethanol was added, and samples were electrophoresed on a 12.5% gel and stained with Coomassie blue. Destained gels were dried and the A_{tl} fragment generated under various conditions was quantified by laser densitometry (full-scale deflection, 0.4 absorbance unit). Panel A, ETA; panel B, ETA-E553D.

Discussion

Photolabeling of Glu-553 of ETA with NAD radiolabeled in the nicotinamide moiety localized Glu-553 to the active site, and the probable structure of the protein-linked photoproduct suggested that the side-chain carboxyl of Glu-553 was close to the bond within NAD ruptured during ADP-ribosyl transfer (Carroll & Collier, 1987). The notion that Glu-553 lies at the active site is supported by X-ray crystallographic studies showing that Glu-553 lies in a major cleft in which NAD and NAD partial structures bind (Allured et al., 1986; Brandhuber et al., 1988). The studies described in the current report were

undertaken to refine and augment findings reported earlier on the effects of the conservative E553D mutation on enzymic and cytotoxic activities of cloned ETA (Douglas & Collier, 1987).

We obtained no indication of major conformational alterations in ETA-E553D relative to the wild-type toxin. Both forms of the toxin were stable during purification from *E. coli* extracts and storage, and the purified proteins were virtually indistinguishable from each other (and from ETA produced by *P. aeruginosa* PA103) with respect to molecular weight, near-UV absorption and fluorescence spectra, immunoreactivity, and substrate affinity (see below). ETA-E553D did elute slightly later from a phenyl-Sepharose column and showed greater sensitivity to thermolysin digestion when the activated whole toxin was treated with this enzyme in the presence of NAD. These changes could reflect only a slight conformational alteration.

Although the proenzymic nature of ETA implies that the native toxin undergoes processing in vivo, the molecular species that actually catalyzes the ADP-ribosylation of EF-2 within sensitive cells is unknown. We performed detailed measurements of kinetic parameters on the A_{t1} fragments and obtained results consistent with data from activated whole toxin (although the A_{t1} fragment showed a 3–5-fold higher specific activity). We assume that the enzymically active molecular species generated in vivo, whatever its nature, shows similar properties.

The most dramatic differences between ETA-E553D and the wild-type toxin were in cytotoxicity (400 000-fold), as measured by inhibition of protein synthesis in L-M cells, and in NAD:EF-2 ADP-ribosyltransferase activity (3000-fold). A somewhat smaller reduction was observed in NAD glycohydrolase activity (50-fold). Activity as a substrate for photoaffinity labeling with radiolabeled NAD was reduced by only about 2-fold.

The changes in ADP-ribosylation activity observed in the E553D mutant could result from alterations in substrate affinity, catalytic events, or both. Alterations in K_M values for NAD or EF-2 were 2-fold or less, and the K_D for NAD was unchanged, as measured by quenching of protein fluorescence. Whereas substrate affinity was virtually unaffected, k_{cat}, in contrast, was reduced by a factor of ca. 4000. These findings are consistent with the notion that Glu-553 may serve a catalytic role in the ADP-ribosylation of EF-2. The effects of mutating Glu-148 of DT to Asp were qualitatively similar, but somewhat less dramatic (Tweten et al., 1985).

The role of Glu-553 is unknown, but current evidence indicates that the side-chain carboxyl function is important. Studies in which ETA containing Gln at position 553 was produced in *E. coli* showed no ADP-ribosylation activity in the product (M. Lukac and R. J. Collier, unpublished results). In addition, carboxymethylation of ETA containing Cys substituted at position 553 restored ADP-ribosylation activity from a level 16 000-fold below that of wild type to a level equivalent to 1/6 that of wild type; carboxamidomethylation caused no increase in activity (Lukac & Collier, 1988). Thus the carboxyl group cannot be functionally replaced by a carboxamide. No data are yet available regarding the pK_a values of Glu-553 in ETA or Glu-148 in DT or of Asp residues substituted at these positions.

The finding that the E553D substitution inhibited NAD glycohydrolase activity to a lesser degree than ADP-ribosylation of EF-2 is similar to an observation in DT, in which the E148D mutation causes only a decline of less than 2-fold (K. Reich, B. Wilson, B. Weinstein, and R. J. Collier, unpublished results). Other mutations in DT in which Gln or Ser have been substituted for Glu-148 also show only slight diminution in this activity. Thus catalysis of NAD glycohydrolase activity, a slow nonphysiological side reaction, may be by a different catalytic mechanism, in which the carboxyl function of these glutamic acid residues is relatively unimportant.

The ability of ETA-E553D to intoxicate cells was affected even more drastically than ADP-ribosylation of EF-2. When protein synthesis was assayed in mouse L-M cells after a 5-h incubation with wild-type or mutant toxin, no inhibition was observed with concentrations of mutant toxin 400 000-fold greater than the concentration of wild-type ETA giving 50% inhibition. The fact that the change in measured cytotoxic activity resulting from the E553D mutation was at least 2 orders of magnitude greater than the alteration in ADP-ribosylation activity may result from any, or a combination, of several factors. Toxicity measurements are dependent on many variables, including the duration of incubation, and no absolutely standard set of conditions has been defined for measurements in vitro. A 4000-fold change in k_{cat} alone might give rise to a much greater change in the instantaneous rate of protein synthesis at a given point in time, depending on factors such as the ratio of EF-2 to ribosomes. This would affect the fraction of EF-2 that must be inactivated to achieve a given percentage inhibition of protein synthesis.

The extent of ADP-ribosylation of EF-2 would be expected to depend upon stability of the toxin to attack by proteases or elements of the protein turnover machinery at various steps in the intoxication process. Evidence presented here that the E553D A_{t1} fragment–NAD complex is more sensitive to attack by thermolysin than the corresponding wild-type A_{t1} complex suggests that this factor may contribute to the large differences observed in cytotoxicity. While it is conceivable that other steps in intoxication—receptor binding and membrane translocation—may be affected by the mutation, it would be surprising to detect large effects on these functions, since the sites responsible for these functions reside on other folding domains. In agreement with this notion, experiments with ETA-E553D in crude extracts indicated that the mutant toxin competed with ETA for receptor binding (Douglas & Collier, 1987).

The present study supports the concept that Glu-553 of ETA is functionally similar to or identical with Glu-148 of DT. Not only do both residues undergo the same photoaffinity labeling reaction with NAD, but substitution of either one with Asp alters biochemical properties in a qualitatively similar manner. As shown here for the E553D mutation, E148D in DT dramatically affected k_{cat} of the NAD:EF-2 ADP-ribosyltransferase reaction without significantly affecting substrate affinity, reduced NAD glycohydrolase activity much less dramatically, and reduced, but did not eliminate, photoaffinity labeling with NAD.

Moreover, despite the major differences between ETA and DT in primary structure and order of functional domains within the molecule, there is significant sequence similarity within the respective enzymic domains (Carroll & Collier, 1988; Zhao & London, 1988; Gill, 1988; Brandhuber et al., 1988). It is likely that Glu-553 of ETA is evolutionarily homologous, as well as functionally similar, to Glu-148 of DT. Although it remains unknown whether other toxins, and other nontoxic enzymes, of the ADP-ribosyltransferase family have functionally similar Glu residues, it has been shown recently that Glu-129 of the S-1 subunit of pertussis toxin can be photolabeled with NAD, and mutations at this site cause

dramatic reductions in enzymic activity (Barbieri et al., 1989; Cockle, 1989).

The information obtained by studies of active sites of bacterial toxins has potential applications in the development of new vaccines by recombinant DNA methods, and the glutamic acid residues identified so far represent obvious targets. Lukac and Collier deleted residue 553 of ETA, yielding a protein that was apparently devoid of enzymic and cytotoxic activities while retaining immunogenicity (Lukac et al., 1988); a similar mutant of DT, in which Glu-148 has been deleted, is under study. Mutations of pertussis toxin S-1 subunit, in Glu-129 as well as other residues, are also being intensively studied with a view toward creating a novel acellular vaccine against whooping cough (Barbieri et al., 1989; Pizza et al., 1989).

Acknowledgments

We thank Steve Carroll and Joe Barbieri for helpful discussions, Karl Reich for providing purified EF-2, and Inger-Helene Madshus and Brenda Wilson for help with certain experiments.

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