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Pseudomonas aeruginosa Exotoxin A: Effects of Mutating Tyrosine-470 and Tyrosine-481 to Phenylalanine†

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ABSTRACT: Directed mutagenesis was used to probe the functions of Tyr-470 and Tyr-481 of *Pseudomonas aeruginosa* exotoxin A (ETA) with respect to cytotoxicity, ADP-ribosylation of elongation factor 2 (EF-2), and NAD-glycohydrolase activity. Both of these residues lie in the active site cleft, close to Glu-553, a residue believed to play a direct role in catalysis of ADP-ribosylation of EF-2. Substitution of Tyr-470 with Phe caused no change in any of these activities, thus eliminating the possibility that the phenolic hydroxyl group of Tyr-470 might be directly involved in catalysis. Mutation of Tyr-481 to Phe caused an approximately 10-fold reduction in NAD:EF-2 ADP-ribosyltransferase activity and cytotoxicity but no change in NAD-glycohydrolase activity. The latter mutation did not alter the K_M of NAD in the NAD-glycohydrolase reaction, which suggests that the phenolic hydroxyl of Tyr-481 does not participate in NAD binding. We hypothesize that the phenolic hydroxyl of Tyr-481 may be involved in the interaction of the toxin with substrate EF-2.

Exotoxin A (ETA), the most toxic protein produced by *Pseudomonas aeruginosa*, acts by an enzymic mechanism similar to that of diphtheria toxin (DT). Domain III, the carboxyl-terminal folding domain defined in the crystallographic structure of Allured et al. (1986, 1987), shows sequence homology with the catalytic fragment of DT (fragment A) (Carroll & Collier, 1988; Brandhuber et al., 1988; Zhao & London, 1988), and like fragment A, domain III catalyzes transfer of the ADP-ribosyl moiety of NAD to elongation factor 2 (EF-2). Thereby, EF-2 is inactivated and protein synthesis of sensitive eukaryotic cells is inhibited.

Glu-553 of ETA and Glu-148 of DT were identified by photoaffinity labeling as functionally homologous active site residues (Carroll & Collier, 1987). Results of site-directed

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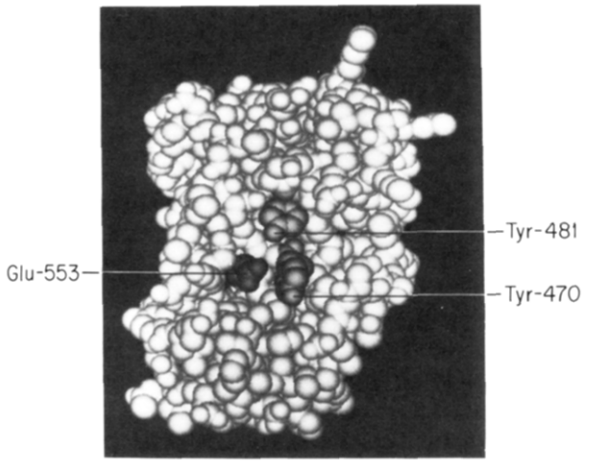

FIGURE 1: A view of the active site cleft of exotoxin A, with Glu-553, Tyr-470, and Tyr-481 highlighted. The model shown was generated from the crystallographic model of Allured et al. (1986), with the Promodeler I molecular graphics program (New England BioGraphics, Peacham, VT). Atomic coordinates were provided by David McKay.

mutagenesis support the notion that these residues play a key role in catalysis (Tweten et al., 1985; Douglas & Collier, 1987). In ETA Glu-553 is located within a prominent cleft of domain III, which, by independent crystallographic evidence, contains the NAD binding site (Allured et al., 1987; Brandhuber et al., 1988). Current data leave major uncertainties, however, about the conformation of bound NAD and about residues that directly contact the ligand.

There are several aromatic residues in the general vicinity of Glu-553 in the crystallographic model of Allured et al. (1987). The most prominent of these (Figure 1) are two tyrosine residues, Tyr-470 and Tyr-481, both of which are conserved in our primary structure alignment with diphtheria toxin (the homologous residues in diphtheria toxin being Tyr-54 and Try-65, respectively; Carroll & Collier, 1988). To probe the possible functions of these residues in substrate binding and catalysis, we undertook directed mutagenesis studies in which each Tyr was replaced independently with Phe. The results of these studies are reported here.

Materials and Methods

Bacterial Strains and Plasmids. *Escherichia coli* JM103 and M13mp19 were purchased from P-L Biochemicals. pCDPT2, the pBR322 derivative containing the ETA gene downstream of the *tac* promotor, was previously described (Douglas et al., 1987). All genetic manipulations were performed according to Maniatis et al. (1982), with restriction enzymes and T4 DNA ligase from New England Biolabs.

Oligonucleotide-Directed Mutagenesis. The mutagenic oligonucleotides 5′-GCCGGCGATGAAGAAACCGCG-3′ and 5′-CCTGGGCGAAGCCGTAGGCC-3′ that are complementary to nucleotides 2219–2239 and 2251–2270, respectively, were synthesized on an Applied Biosystems 381A DNA synthesizer and purified by high-performance liquid chromatography. The mutagenesis was performed with the Amersham oligonucleotide-directed mutagenesis system according to the manufacturer's handbook [see also Douglas et al. (1987) and Douglas and Collier (1987)]. The 5′-phosphorylated oligonucleotides were annealed to single-stranded DNA prepared from M13mp19 carrying the 553-bp *Sal*I-*Bam*HI fragment from pCDPT2. Predominantly homoduplex RF molecules were transformed into *E. coli* JM103. M13 bacteriophage in supernatants were screened for mutants by slot blotting (Douglas & Collier, 1987). Positive clones were plaque purified, and mutations were verified by dideoxy sequencing the [α-^{32}P]ATP (800 Ci/mmol) and reagents from Amersham as recommended by the supplier. The 460-bp *Bam*HI–*Eco*RI fragment from pCDPT2 was then cloned in the corresponding sites of the mutagenized M13 RF DNA. This enabled us to excise the 279-bp *Sac*II–*Xho*I fragment containing either mutation from the recombinant M13 RF DNA and insert it into pCDPT2 that was pretreated with calf intestinal phosphatase (Boehringer), replacing thus the corresponding wild-type fragment. The new plasmids were named pCDPTY470F and pCDPTY481F and were transformed into *E. coli* JM103. A *Sal*I–*Bam*HI fragment from either plasmid was recloned into M13mp19, and dideoxy DNA sequencing was employed to confirm the desired mutations in the expression vectors.

Production and Analysis of Recombinant ETA. Growth of *E. coli* JM103 with pCDPTY470F or pCDPTY481F and preparation of bacterial extracts were carried out as previously described (Douglas et al., 1987). Total protein was quantified by the method of Bradford (1976) with protein assay reagent from Pierce and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970). ETA-cross-reactive material was examined by immonoblotting and quantified by radioimmunoassay according to Douglas and Collier (1987). ADP-ribosyltransferase activity and cytotoxicity of bacterial extracts were tested as previously reported with authentic ETA of *P. aeruginosa* PA103 as standard (Douglas & Collier, 1987). Inhibition constants (K_i) for adenine, adenosine, and nicotinamide were determined in ADP-ribosylation assays, inhibitor concentrations varying from 0 to 2 mM. NAD-glycohydrolase activity of recombinant ETA in bacterial extracts was assayed in 0.05 M Tris, pH 7.8, with 30 μM BSA and 2 μM [4-^{3}H]NAD (2.2 Ci/mmol, Amersham) for 17 h at 37 °C. The reaction was stopped by adding $NaBO_4$, pH 8.0, to 150 mM, and radiolabeled nicotinamide was extracted with water-saturated ethyl acetate for liquid scintillation counting.

Results

We created the Y470F and Y481F mutant forms of cloned ETA by a protocol that involves a strand-specific selection step and generates predominantly homoduplex mutant DNA (Taylor et al., 1985). Mutagenesis efficiencies were >85%. DNA sequence analysis confirmed that the expression vectors, pCDPTY470F and pCDPTY481F, each contained the desired mutation and that no second-site mutations occurred within the mutagenized fragment. *E. coli* JM103 expressing Y470F-ETA or Y481F-ETA produced comparable amounts of immunoreactive material (85–120 pmol equiv of ETA/mg of protein, corresponding to about 1% of total protein) (Table I). Both of the mutant toxins were stable proteins, comigrating with authentic ETA from *P. aeruginosa* (Figure 2). No immunoreactive degradation products or loss of immunoreactive material was detected in any of the extracts, even after storage at −20 °C for several months.

Activated ETA transfers the ADP-ribose moiety of NAD to EF-2 (ADP-ribosylation) or H_2O (NAD-glycohydrolysis). We measured the effects of the two Y → F substitutions on both enzymic activities and on cytotoxicity. As demonstrated earlier, ADP-ribosyltransferase and cytotoxic activities of recombinant ETA may be accurately measured in crude bacterial extracts (Douglas & Collier, 1987; Lukac & Collier, 1988). We found that this is also true for NAD-glycohydrolase activity; extracts of *E. coli* JM103 without ETA exhibited only background levels of this activity.

Specific NAD:EF-2 ADP-ribosyltransferase activity (enzymic activity per unit of cross-reactive material) measured

Table I: Properties of Bacterial Extracts Containing Y470F- or Y481F-ETA

	activity, relative to that of JM103(pCDPT)	
	JM103-(pCDPT-Y470F)	JM103-(pCDPT-Y481F)
immunoreactivity (I)[a]	0.89	0.98
ADP-ribosyltransferase activity (II)[a]	0.87	0.070
specific ADP-ribosyltransferase activity (II/I)	0.97	0.071
cytotoxicity (III)[a]	0.88	0.098
specific cytotoxicity (III/I)	0.99	0.100
NAD-glycohydrolase activity (IV)[a]	1.08	0.82
specific NAD-glycohydrolase activity (IV/I)	1.20	0.84

[a] Extracts from *E. coli* JM103(pCDPTY470F) and JM103-(pCDPTY481F) were assayed for total protein, immunoreactive material, ADP-ribosyltransferase activity, cytotoxic activity to mouse LM929 cells, and NAD-glycohydrolase activity. The activities I, II, III, and IV are listed as fractions of the activities (per unit of protein) obtained from extracts of JM103(pCDPT2) expressing wild-type ETA, which were defined as 1.0. The results shown are the average of at least three independent experiments (standard deviation ≤±20%). Activities of recombinant wild-type ETA were derived from standard curves prepared with authentic ETA and were 115, 120, 186, and 155 pmol equiv of ETA/mg of protein of extract for immunological, ADP-ribosyltransferase, cytotoxic, and NAD-glycohydrolase activity, respectively.

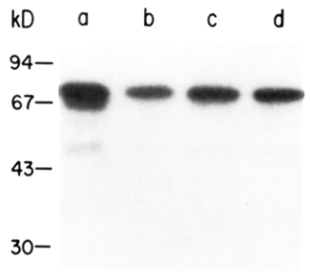

FIGURE 2: Western blot of extracts of *E. coli* JM103 with pCDPT2, pCDPTY470F, or pCDPTY481F. The proteins were separated on a 11.25% SDS–polyacrylamide gel, transferred to a nitrocellulose filter, and probed with ^{125}I-labeled rabbit anti-ETA IgG (Douglas et al., 1987). Authentic ETA from *P. aeruginosa* PA103 (a) and extract of *E. coli* JM103 with pCDPT2 (b), with pCDPTY470F (c), and with pCDPTY481F (d).

in bacterial extracts was unaffected by the Y470F mutation (Table I), and cytotoxicity was also unaffected. In contrast, the Y481F mutation caused approximately 10-fold reduction in specific ADP-ribosyltransferase activity and cytotoxicity. Neither of the two mutations caused a significant change in NAD-glycohydrolase activity (Table I; Figure 3).

As a means of estimating relative affinities of NAD for the mutant and wild-type toxins, we determined values of K_m of the dinucleotide in the NAD-glycohydrolase reaction. NAD concentrations were varied from 2 to 64 μM. Values for V_{max} confirmed that the NAD-glycohydrolase activities of the three proteins are similar (7.5×10^{-12}, 8.1×10^{-12}, and 5.4×10^{-12} mol/h for wild type, Y470F-ETA, and Y481F-ETA, respectively). Measurement of slopes in Woolf–Augustinsson–Hofstee plots (V vs $V/[S]$) (Figure 3) gave K_m values of 19, 15, and 13 μM NAD for wild type, Y470F-ETA, and Y481F-ETA, respectively. These results support the notion that the 10-fold decrease in NAD:EF-2 ADP-ribosyltransferase activity observed in Y481F-ETA is not due to altered affinity for NAD.

We also measured K_i values of adenine, adenosine, or nicotinamide in the NAD:EF-2 ADP-ribosyltransferase reaction with Y481F- and Y470F-ETA. The values obtained did not

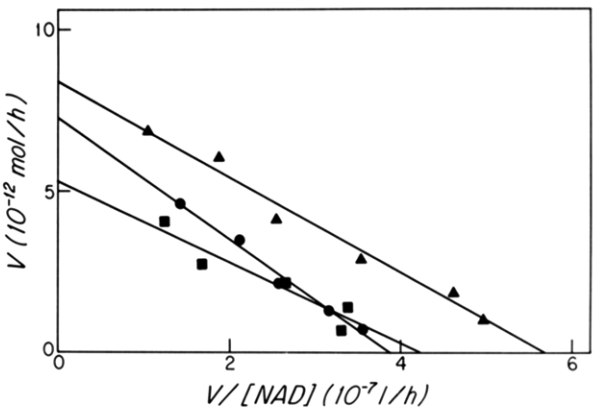

FIGURE 3: Determination of Michaelis–Menten constants (K_m) of NAD for Y470F-ETA, Y481F-ETA, and wild-type ETA. NAD-glycohydrolase assays were performed in the presence of varying concentrations of NAD (2–64 μM) as described under Materials and Methods. Velocities (V) were plotted versus $V/[NAD]$ and analyzed by linear regression. The K_m values, which correspond to the negative slopes, are given in parentheses: (●) extract with wild-type ETA (19 μM); (▲) extract with Y470F-ETA (15 μM); (■) extract with Y481F-ETA (13 μM).

Table II: Concentrations of Adenine, Adenosine, and Nicotinamide Required for Half-Maximal Inhibition of ADP-Ribosylation of EF-2, with Bacterial Extracts Containing Y470F-ETA, Y481F-ETA, or Wild-Type ETA as Source of Enzyme[a]

	K_i (μM)		
	adenine	adenosine	nicotinamide
wild-type ETA	116	380	318
Y470F-ETA	125	365	400
Y481F-ETA	147	340	379

[a] Standard deviations were ±25% or less.

differ substantially from those with wild-type ETA (Table II), thereby supporting the notion that neither substitution resulted in a major alteration in affinity for NAD.

Discussion

Photoaffinity labeling and directed mutagenesis studies have implicated Glu-553 in ETA and its functional homologue in diphtheria toxin, Glu-148, as active site residues that may play a direct role in catalysis. Ultraviolet irradiation of NAD complexes of enzymically active forms of ETA and DT leads to transfer of the nicotinamide moiety to these glutamic acid residues (Carroll & Collier, 1984, 1987). This suggests close proximity, or direct contact, of the nicotinamide moiety with these Glu residues in the enzyme–dinucleotide complex.

Tyr-470 and Tyr-481 were chosen as potentially important residues for interactions with NAD and EF-2 because they are in the active site cleft, close to Glu-553, in the 3.0-Å X-ray crystallographic structure of native ETA. In the absence of evidence to the contrary, we have assumed that conversion of the native ETA to the enzymically active form does not cause major alterations of active site structure.

While the current studies were under way, Brandhuber et al. (1988) proposed a model of NAD binding, based on biochemical, crystallographic, and computer modeling studies, which involves specific interactions of bound NAD with both Tyr-470 and Tyr-481. Crystals soaked in NAD did not yield a difference density that could be interpreted as a complete NAD molecule; but difference Fourier maps with bound adenine, AMP, or ADP indicated that the adenine ring of these compounds interacts with the phenolic ring of Tyr-481. This strongly suggests that the adenine moiety of NAD binds at the same site. Iodination of ETA under conditions in which Tyr-481 was the only Tyr modified in the enzymic domain

abolished ADP-ribosyltransferase activity, and it was proposed that iodination could inhibit enzymic activity by sterically hindering interaction with the adenine moiety of substrate NAD.

In the model of Brandhuber et al. (1988) the nicotinamide ring of NAD is stacked on the indole ring of Trp-466 (corresponding to Trp-50 in DT), and the phenolic hydroxyl of Tyr-470 is positioned near the nicotinamide–ribose bond broken during catalysis. The model therefore raises the possibility that the Tyr-470 phenolic hydroxyl might be involved in catalysis.

Since mutating Tyr-470 to Phe caused no significant change in either NAD:EF-2 ADP-ribosyltransferase activity or NAD-glycohydrolase activity, a role for the Tyr-470 phenolic hydroxyl in catalysis is effectively eliminated. The Tyr-470 phenolic ring may, nonetheless, play a structural role in substrate binding. The ring might provide a contact surface for the nicotinamide ring of NAD and position the nicotinamide moiety adjacent to the Glu-553 side chain. This would provide an explanation for the highly specific photolabeling of Glu-553 observed, in which the nicotinamide ring of NAD is transferred into covalent linkage with the γ-methylene group of this glutamic acid.

When Phe was substituted for Tyr-481, interactions with bound NAD or NAD partial structures were essentially unaffected. NAD-glycohydrolase activity and the value of K_m for NAD in this reaction were virtually unchanged, as were values of K_i for adenine, adenosine, and nicotinamide in the ADP-ribosylation of EF-2. This implies that the phenolic hydroxyl group of Tyr-481 plays little or no role in NAD binding; hydrophobic interactions therefore apparently predominate in this interaction.

Nonetheless, Y481F-ETA showed about 10-fold lower activity than wild-type ETA in ADP-ribosylating EF-2. This suggests that the phenolic hydroxyl of Tyr-481 has an effect on EF-2 binding, or on some other aspect of the enzymic process. Studies to measure the K_m of EF-2 are under way. The phenolic hydroxyl of Tyr-481 might affect reaction rate by any of a number of alternative mechanisms, such as by altering the orientation of the attacking diphthamide residue of EF-2. Iodination of Tyr-481 might inhibit ADP-ribosylation of EF-2 by altering interactions of this residue with NAD, as suggested by Brandhuber et al. (1988), with EF-2, or with both.

The fact that specific cytotoxicity is approximately proportional to NAD:EF-2 ADP-ribosyltransferase activity in Y481F- and Y470F-ETA implies that neither of these mutations significantly affects the binding or transport functions of the toxin, or the stability of the enzymically active moiety within the cell. The finding that NAD-glycohydrolase activity was not affected to the same extent as NAD:EF-2 ADP-ribosyltransferase activity in Y481F-ETA is consistent with other results in our laboratory suggesting important differences in catalytic mechanism between the two reactions (Douglas and Collier, unpublished data; Reich, Weinstein, and Collier, unpublished data). Finally, we predict, on the basis of sequence homology, that mutation of Tyr-54 and Tyr-65 of diphtheria toxin to Phe would have similar effects to the mutations of Tyr-470 and Tyr-481, respectively, described here.

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Registry No. Tyr, 60-18-4; EF-2·ADP-ribosyltransferase, 52933-21-8; NAD-glycohydrolase, 9032-65-9.

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