A Fluorescence Investigation of the Active Site of *Pseudomonas aeruginosa* Exotoxin A*

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Single tryptophan mutant proteins of a catalytically active domain III recombinant protein (PE24) from *Pseudomonas aeruginosa* exotoxin A were prepared by site-directed mutagenesis. The binding of the dinucleotide substrate, NAD⁺, to the PE24 active site was studied by exploiting intrinsic tryptophan fluorescence for the wild-type, single Trp, and tryptophan-deficient mutant proteins. Various approaches were used to study the substrate binding process, including dynamic quenching, CD spectroscopy, steady-state fluorescence emission analysis, NAD⁺-glycohydrolase activity, NAD⁺ binding analysis, protein denaturation experiments, fluorescence lifetime analysis, steady-state anisotropy measurement, stopped flow fluorescence spectroscopy, and quantum yield determination. It was found that the conservative replacement of tryptophan residues with phenylalanine had little or no effect on the folded stability and enzyme activity of the PE24 protein. Dynamic quenching experiments indicated that when bound to the active site of the enzyme, the NAD⁺ substrate protected Trp-558 from solvent to a large extent but had no effect on the degree of solvent exposure for tryptophans 417 and 466. Also, upon substrate binding, the anisotropy of the Trp-417(W466F/W558F) protein showed the largest increase, followed by Trp-466(W417F/W558F), and there was no effect on Trp-558(W417F/W466F). Furthermore, the intrinsic tryptophan fluorescence exhibited the highest degree of substrate-induced quenching for the wild-type protein, followed in decreasing order by Trp-417(W466F/W558F), Trp-558(W417F/W466F), and Trp-466(W417F/W558F). These data provide evidence for a structural rearrangement in the enzyme domain near Trp-417 invoked by the binding of the NAD⁺ substrate.

*Pseudomonas aeruginosa* is a ubiquitous, Gram-negative, opportunistic pathogen that is commonly found in soil, water, sewage, and even in hospital environments (1). This bacterium is a leading cause of infections in burn, cystic fibrosis, and postoperative patients and in other various immune-compromised hosts. *P. aeruginosa* synthesizes a number of extracellular toxic products believed to be involved in the pathogenesis of these infections. The most toxic factor secreted by *P. aeruginosa* is the 66-kDa protein exotoxin A (ETA). ETA belongs to a family of toxins related in their mechanisms of action, which include diphtheria toxin (DT), pertussis toxin, cholera toxin, and *Escherichia coli* heat-labile toxin. These toxins exert their effects via ADP-ribosylation of specific target molecules within eukaryotic cells. More specifically, ETA catalyzes the transfer of the ADP-ribos moiety of NAD⁺ onto elongation factor 2 (2, 3). This covalent transfer inactivates eukaryotic elongation factor 2, rendering it incapable of polypeptide chain elongation, inhibiting protein synthesis, and eventually killing the target cell.

The three-dimensional crystallographic structure of the 613-residue ETA protein has been solved to a resolution of 3.0 Å (4). Based on the crystal structure of the 66-kDa ETA molecule, along with other physicochemical data, molecular models were proposed that consist of three distinct functional domains (5). Domain Ia (residues 1–252) is involved in receptor binding, domain Ib (residues 365–404) has no known function, and domain II (residues 253–364) aids in translocation across the endoplasmic reticulum membrane into the host cell cytoplasm after receptor-mediated endocytosis. Domain III (residues 405–613) comprises the catalytic domain, which contains an extended cleft postulated to be the active site of the enzyme.

From the resolved 2.3-Å crystal structure of DT (6), some active site residues are located in essentially identical positions within ETA and DT. Previously, a model was proposed in which NAD⁺ fits into the cleft with the adenine ring bound in the hydrophobic pocket defined by the aromatic rings of Tyr-470 (Tyr-54, DT), Tyr-481 (Tyr-65, DT), Trp-466 (Trp-50, DT), Trp-558 (Trp-153, DT), and His-440 (His-21, DT) (7). The model suggests that either the nicotinamide or adenine ring of NAD⁺ would stack on the indole ring of Trp-466. More recently, the 2.5-Å crystal structure of domain III of ETA complexed with NAD⁺ hydrolysis products (AMP and nicotinamide) has been solved (8), and close inspection of this structure revealed that NAD⁺ likely does not directly interact with the Trp-466 side chain (9). Furthermore, a three-dimensional structure of domain III of ETA complexed with the NAD⁺ analogues, β-TAD⁺, has also been determined (10), as has the structure of DT complexed with intact NAD⁺ (11). Bell et al. (12) compared the conformations of NAD(P)⁺ bound to 23 distinct NAD(P)⁺-binding oxidoreductases enzymes. The majority of the oxidoreductase-bound NAD(P)⁺ conformations were found to be similar, but the conformation of NAD⁺ bound to DT (and ETA) was 1

1 The abbreviations used are: ETA, *P. aeruginosa* exotoxin A; ave, average; β-TAD⁺, β-methylene-thiazole-4-carboxamide adenosine dinucleotide; DT, diphtheria toxin; e-AMP, L,N'-etheno-AMP; εm, molar extinction coefficient; ε-NAD⁺, L,N'-etheno-NAD⁺; GH, NAD⁺ glycohydrolase; Gn-HCl, guanidine hydrochloride; λmax, fluorescence emission maximum; NATA, N-acetyltryptophanamide; PE24, *P. aeruginosa* exotoxin A 24-kDa C-terminal fragment; Qf, fluorescence quantum yield; WT, wild-type.
found to be unusual. This difference was seen in the highly folded conformation of the nicotinamide mononucleotide portion of NAD$^+$ and a strained N-glycosidic bond, which places the nicotinamide ring outside the preferred anti and syn conformations. In DT and ETA, the NAD$^+$-binding site is formed at the junction of the two anti-parallel $\beta$-sheets that are orthogonal to one another. This is again in sharp contrast to other NAD(P$^+$) enzymes that bind NAD(P$^+$) at the C-terminal end of a parallel $\beta$-sheet.

Within domain III of ETA are three tryptophans (Trp-417, Trp-466, and Trp-558) that could potentially participate in hydrophobic stacking interactions as described previously. Previously, we demonstrated that upon NAD$^+$ binding to the catalytic domain, the intrinsic protein fluorescence of ETA is significantly quenched (9). This effect was attributed to the fluorescence quenching of one or more tryptophans located within domain III. However, at that time, a catalytically active fragment of ETA that consisted of domain III only was not available. In order to elucidate the roles of these tryptophans in substrate binding and catalysis, we made conservative substitutions (phenylalanine for tryptophan) within PE24, a recombinant protein that is a catalytically active fragment of ETA consisting primarily of domain III, using a site-directed mutagenesis technique. The effects of these mutations were determined by accessibility of the Trp residues to acrylamide, CD spectroscopy, NAD$^+$-glycodydrolyase (GH) activity, NAD$^+$ binding affinity, protein folded stability, fluorescence lifetimes, anisotropies, and quantum yields ($Q_\beta$). The results suggest that Trp-558 is protected from the solvent when NAD$^+$ fills the active site and that a major structural change occurs within the catalytic domain within the vicinity of Trp-417.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Enzymes**—Restriction endonucleases, T7 DNA polymerase, and T4 DNA ligase were purchased from Amersham Pharmacia Biotech (Baie D’Urfé, Quebec, Canada) and New England Biolabs (Mississauga, Ontario, Canada). The BL21(DE3) cells were obtained from Novagen (Madison, WI); isopropyl $\beta$-D-thiogalactopyranoside was from Alexis Corp. (San Diego, CA); Q-Sepharose Fast-Flow anion exchange and agarose-agarose resins were from Amersham Pharmacia Biotech. Dithiothreitol was from Promega Corp. (Madison, WI). The following chemicals were obtained from Sigma: NAD$^+$, TRIZMA base, bovine serum albumin, 1M$^\text{--}$$\text{etheno-AMP (e-AMP)}$, and 1M$^\text{--}$$\text{etheno-}\text{nicotinamide adenine dinucleotide (e-NAD$^+$)}$. SequanaTM grade of both guanidine hydrochloride (Gn-HCl) and urea were obtained from Pierce, and imidazole was obtained from Fluka (Buchs, Switzerland).

**Purification of PE24**—PE24, the C-terminal catalytic fragment of P. aeruginosa exotoxin A, was overexpressed in E. coli using the plasmid pPE24-5-399, in which a poly-His sequence was introduced into the protein at the C terminus by recombinant DNA methods. The His$_\alpha$-tagged protein was then purified from BL21 (DE3) cells as described previously for PE40 (9) except that the esmotic shock lysis solution from 500 ml of induced cell culture in 20 mM Tris-HCl, pH 7.9, was passed through a 2 ml cholate-agarose affinity column charged with 50 mM NiSO$_4$. The column was washed with 20 ml Tris-HCl, pH 7.9, buffer containing 50 mM NaCl (Buffer A) and 5 mM imidazole (5 ml) followed by washing with 10 ml of Buffer A at a higher imidazole concentration (60 mM). PE24 protein possessing a poly-His sequence was eluted from the column with 8 ml of Buffer A containing 250 mM imidazole, the samples were collected in 1 ml fractions, and a yield of 5 mg of protein was routinely obtained. The appropriate fractions were pooled and exhaustively dialyzed against 20 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaCl.

**Oligonucleotide-directed Mutagenesis**—Mutagenesis was performed using the Kunkel method as described previously (13). Deoxyo sequencing was performed using an ABI Prism model 377 DNA sequencer using dye termination and cycle sequencing. DNA samples were analyzed in 4.5% acrylamide gels that were 36 cm in length.

**Spectroscopic Measurements**—Steady-state fluorescence measurements were performed using a PTI Alphascan spectrophotometer (Photon Technology International, South Brunswick, NJ) equipped with a water-jacketed sample chamber set to 25 °C.

**NAD$^+$-glycodydrolyase Assay**—For the GH assay, the excitation wavelength was 305 nm, the emission monochromator was set to 0 nm, and a 309 nm cutoff filter (Oriel Corporation, Stratford, CT) was placed on the sample chamber side of the emission monochromator to maximize signal detection (4 nm excitation and emission slit widths). Buffer (20 mM Tris-HCl, pH 7.4, unless otherwise stated) and e-NAD$^+$ (Sigma N-2654) was given various concentrations. Each reaction stock solution, 30 ml prepared in distilled water, $\epsilon^{285 \text{nm}} = 6000 \text{ M}^{-1} \text{cm}^{-1}$) were combined in an ultramicro (3 mm path length) cuvette (Helma Inc., Concord, ON). The cuvette was placed in the sample chamber for 5 min to allow temperature equilibration of the sample. The reaction was started by the rapid addition and mixing of 2.0 $\mu\text{M}$ protein (final concentration) and the reaction progress monitored by the increase in the fluorescence intensity of the fluorescent substrate, e-NAD$^+$. For each concentration of e-NAD$^+$, a corresponding control sample was prepared, in which buffer was added to the cuvette in place of PE24. This control was used to confirm that the increase in fluorescence intensity observed in the presence of PE24 was due to the presence of the enzyme. The water-catalyzed GH activity was determined to be less than 10% of the enzyme-catalyzed reaction (reactions performed in the absence of enzyme).

**Quenching of Intrinsic Protein Fluorescence**—The NAD$^+$-dependent quenching of intrinsic protein fluorescence was monitored as a function of the concentration of NAD$^+$. Duplicate reactions were performed over a concentration range of 0–750 $\mu\text{M}$ NAD$^+$ in the presence of toxin at a concentration of 0.5 $\mu\text{g}$ of 50 mM Tris-HCl. Triplicates were excited at 295 nm (4 nm band pass) and the fluorescence intensity was measured over the range of 305–400 nm (4-nm band pass) in the computer-controlled PTI Alphascan-2 spectrofluorometer. The final concentration of PE24 used in the experiments was 2.5 $\mu\text{M}$. The fluorescence intensity of N-acetyltryptophanamide (NATA) as a function of NAD$^+$ concentration was used to correct for the absorbive screening by NAD$^+$ at the excitation wavelength. The binding constants were calculated using the Scatchard binding analysis, and the data plots were linear corresponding to a single set of NAD$^+$ binding sites.

**Unfolding Conditions and $\Delta G$ Measurements**—The PE24 protein in 100 mM NaCl, 20 mM Tris-HCl buffer (pH 7.4) was mixed with the appropriate volume of 8 $\mu\text{M}$ SequanaTM grade Gn-HCl to provide solutions from 0 to 6 M Gn-HCl and 0.1 $\text{mg/ml}$ protein concentration. Unfolding experiments with urea necessitated the preparation of fresh solutions of a 9 M urea SequanaTM grade stock that was added to the protein solutions to make solutions from 0 to 8 M urea. In all cases, the samples were allowed to equilibrate for 30 min at 25 °C prior to spectrophotometric analysis. Refolding of PE24 was examined by diluting the protein/denaturant solutions with buffer in 0.25 $M$ increments with a 15 min incubation time between dilutions. The spectroscopic unfolding/refolding data were analyzed as described previously (13).

**Kinetic Data Collection**—All kinetic data were obtained at 25 °C and were collected using an Applied Photophysics stopped flow fluorescence spectrometer equipped with both excitation and emission monochromators (Applied Photophysics, Leatherhead, UK) as described previously (14). Excitation slit widths were 4 nm, and emission slit widths were 6 nm for all measurements. The kinetic traces shown are an average of three or more experiments. Changes in Trp fluorescence upon denaturation were monitored by 10:1 mixing of 4.44 $\mu\text{M}$ SequanaTM grade Gn-HCl with the PE24 protein (20 $\mu\text{M}$) in 20 mM Tris-HCl buffer, pH 7.4, to provide a final Gn-HCl concentration of 4 M and a peptide concentration of 2 $\mu\text{M}$. Tryptophan fluorescence was monitored by 295 nm excitation, and emission was detected at right angles at an emission wavelength of 334 nm for WT PE24.

**Kinetic Data Analysis**—Rate constants were calculated by fitting kinetic data by nonlinear least squares analysis (MicroCal Origin; MicroCal Software Inc., Northampton, MA). A fit was deemed acceptable if it provided a fit with random residuals and had reached minimum error in all fitting parameters (standard errors were typically between 5 and 10%). Kinetic traces, which fitted single exponential kinetics, were fit with the following equation,

$$F = F_0 + F_1(1 - \exp[-kt])$$  \hspace{1cm} (Eq. 1)

where $F$ is the fluorescence at time $t$, $F_0$ is the initial fluorescence at time 0, $F_1$ is the fluorescence change during the unfolding reaction, and $k$ is the rate constant. Kinetic traces, which showed double exponential kinetics, were fit with the following equation,

$$F = F_0 + F_1(1 - \exp[-kt_1]) + F_2(1 - \exp[-kt_2])$$  \hspace{1cm} (Eq. 2)

where $F$ and $F_0$ are as described above, and $F_1$ and $F_2$ are the fluorescence changes for the double exponential unfolding reaction, with rate constants $k_1$ and $k_2$, respectively.
Fluorescence Quantum Yield and Lifetime Measurements—Steady-state fluorescence measurements were made with a PTI spectrofluorometer operating in the ratio mode, with unpolarized excitation and an emission polarizer (Glan-Thompson) oriented at 35° to the vertical to eliminate any effects of anisotropy on quantum yield or intensity measurements. The signal from the buffer blank was subtracted from the sample fluorescence, and the spectra were corrected for the wavelength dependence of the instrument response. Fluorescence quantum yields (Q<sub>f</sub>) for each of the proteins were measured at 25 °C, using N-acetyltryptophanamide (pH 7.0) as a standard as described previously (15).

The excitation wavelength was 295 nm, and the emission was scanned from 340 to 450 nm in 0.5-nm increments (excitation and emission monochromator bandpasses were 2 and 4 nm, respectively). The absorbance of the samples at the excitation wavelength was typically less than 0.1. The values reported represent the mean of at least three determinations.

Time-resolved fluorescence measurements were performed using a PTI LaserStrobe model C-72 lifetime fluorometer (Photon Technology International, South Brunswick, NJ). The excitation source was a pulsed nitrogen laser pumping a dye laser with a frequency doubler. The laser operated at 10 Hz, and the detection channel consisted of an emission monochromator with a stroboscopic detector. The data analysis was performed with a 1-to-4 exponential fitting program involving iterative reconvolution and minimization of chi-squared. The excitation wavelength was 295 nm, and the emission was set at 340 nm with 1-nm bandpasses.

Fluorescence Anisotropy Measurements—Steady-state fluorescence anisotropy values were determined at 25 °C in 20 mM Tris-HCl buffer, pH 7.4. The excitation and emission wavelengths were 295 nm (4-nm bandpass) and 340 nm (6-nm bandpass), respectively. The anisotropy measurements were conducted by using a T-format configuration with a Glan-Thompson prism polarizers. The r<sub>0</sub> values were calculated from the following equation: r<sub>0</sub> = I<sub>ex</sub> – G-factor·I<sub>em</sub>/I<sub>ex</sub> + 203-factor·I<sub>em</sub>. The G-factor was determined by measuring both the vertical and horizontal fluorescence emission with horizontal excitation and was calculated using the equation G = I<sub>lh</sub>/I<sub>hh</sub>. These calculations were performed using the built-in anisotropy function within the PTI FELIX<sup>TM</sup> software.

Steady-Volmer Quenching—Quenching experiments were performed using excitation at 295 nm with fluorescence emission set at 340 nm (4-nm bandpasses). Aliquots of 4 mM acrylamide solution were added to protein solution (25 °C) containing 20 mM Tris-HCl buffer, pH 7.4. The fluorescence intensities were recorded for 30 s, and the trace was integrated to give more reproducible data. The data were corrected for dilution effects and for absorptive screening caused by acrylamide (ε<sub>acrylamide</sub> = 0.25 M<sup>-1</sup> cm<sup>-1</sup> at 295 nm) (16). Quenching data were plotted as the ratio of fluorescence in the absence of quencher (F<sub>0</sub>) to the intensity in the presence of quencher (F<sub>q</sub>) as a function of the quencher concentration. The majority of the Stern-Volmer plots were linear, but for those exceptions, only the linear region of the quenching curves was fit by least squares linear regression analysis. The calculated slope was equated to dynamic parameters according to the modified Stern-Volmer equation: F/F<sub>0</sub> = 1 + K<sub>SV</sub>[Q], where K<sub>SV</sub> is equal to K<sub>d</sub>·R<sub>0</sub>·(1 + G-factor·R<sub>0</sub>) (17, 18). Protein concentrations were adjusted to provide an optical density at the excitation wavelength of less than 0.1.

RESULTS

X-ray Structure and Molecular Model of PE24—Fig. 1A shows the primary sequence of the PE24 protein. It contains the first three residues of domain I from whole toxin followed by residues 399–613 from domain III. At the C terminus is the catalytic domain III. The first three residues of domain I from whole toxin followed by residues 399–613 from domain III. At the C terminus is the catalytic domain III. Furthermore, the Q<sub>f</sub> values of the three naturally occurring Trp residues were similar to those of the WT protein, indicative of normally folded proteins (13). Also, CD analysis showed that the percent a-helical content was similar for all the proteins (17 ± 3%; data not shown). It is known that the fluorescence quantum yields measured in proteins reflect the average of a large number of interactions within proteins and is, perhaps, a more sensitive measure of protein folded structure than λ<sub>em</sub> max values or ΔG (folding) measurements (15).

Upon NAD<sup>+</sup> binding to the PE24 protein, there was no detectable shift in the λ<sub>em</sub> max values for either the WT or the single Trp mutant proteins (Table I), indicating no large change in the net polarity of the environment surrounding the three Trp residues within the catalytic domain. However, the NAD<sup>+</sup> substrate did induce a significant change in the quantum yields for all the single Trp mutant proteins and the WT protein as well (Table I).

<sup>2</sup> W-417 refers to the single tryptophan mutant protein that has two mutations, Trp-466 to Phe and Trp-558 to Phe. This mutant protein could also be designated W466F/W558F. This nomenclature also pertains to the mutant proteins designated W-466 and W-558, which are W417F/W558F and W17F/W466F, respectively. Trp<sup>+</sup> is a Trp-deficient mutant protein, W417F/W466F/W558F.
Fluorescence Lifetimes—The time-resolved decay components for the WT and the three single Trp mutants of PE24 are shown in Table I. The WT protein exhibited a short component ($\tau = 1.55$ ns, 40%) and a long (6.56 ns, 60%) decay time component. Each of the single Trp mutant proteins possessed a short decay component near 1 ns and a longer component between 4.7 and 4.9 ns, with the relative amounts of both decay times being similar. Given the differences in the steady-state fluorescence parameters between these proteins, it is surprising that the decay time components were nearly identical. This is reflected in the $\tau_{ave}$ values, with WT (5.88 ns) and the three mutant proteins possessing similar values ranging between 4.0 and 4.4 ns. Again, the differences exist between the WT protein and the single Trp mutant proteins, perhaps reflecting subtle changes upon Trp $\rightarrow$ Phe replacement that are not evident in the folded conformation and in the biochemical function of the catalytic domain.

When NAD$^+$ was bound to the active site, the $\tau_{ave}$ value for the WT PE24 protein decreased from 5.88 to 2.78 ns (Table I). The short decay time for the WT protein changed from 1.55 to 0.82 ns (39.7 to 84.6%, respectively), whereas the long decay time decreased from 6.56 to 4.66 ns (the normalized pre-exponential also decreased to 15.4% from 60.3%). The $\tau_{ave}$ value for the W-417 mutant protein increased slightly from 4.36 to 4.64 ns, and the short and long lifetime components both increased (short component, 0.93 to 1.54 ns and 43.8 to 56.6%, respectively; long component, 4.88 to 5.72 ns and 56.2 to 43.4%, respectively). There was little or no change in the $\tau_{ave}$ value for the W-466 mutant protein upon substrate binding (4.12 to 3.96 ns) and there was also little change in either decay time component for this protein (Table I). The W-558 mutant protein showed a slight increase in its $\tau_{ave}$ value upon substrate binding and both decay time components increased (short compo-
The deployment of the Trp-deficient (Trp–) mutant (W417F/W466F/W558F) PE24 (Fig. 3). This mutant protein possesses normal GH activity, indicating that the replacement of the three Trp residues within the catalytic domain does not inhibit the ability of the protein to bind the NAD+ substrate. The fluorescence quench data shown in Fig. 3 were surprising in that the W-417 mutant exhibited significant quenching of its lone Trp-417 residue (63%). This indicates that NAD+ must induce a significant structural change within the catalytic domain that results in an altered chemical environment for Trp-417. Also, this mutant was able to bind NAD+, similar to the WT protein (Table II). The W-466 mutant protein also bound NAD+ with affinity comparable to the wild-type protein (Table 1), but the fluorescence of its Trp residue was not quenched to the same extent as for the WT or W-417 proteins (Fig. 3). The W-558 protein showed a reduction in its affinity for the NAD+ substrate (296 and 47 μM for the W-558 and WT proteins, respectively; Table II). However, the level of fluorescence quenching was higher than for W-466 but lower than either the W-417 or WT proteins (Fig. 3 and Table II).

### Table I

| Protein   | NAD+ | Qe (a) | λmax (nm) | α1 (ns) | τ1 (ns) | α2 (ns) | τ2 (ns) | rave (f) | rsav (f) |
|-----------|------|--------|-----------|---------|---------|---------|---------|----------|----------|
| Wild-type | −    | 0.169  | 333       | 0.397   | 1.55    | 0.603   | 5.88    | 0.118    | 0.017    |
|           | +    | 0.045  | 333       | 0.846   | 0.82    | 0.154   | 4.66    | 2.78     | 0.008    |
| W-417     | −    | 0.100  | 332       | 0.438   | 0.93    | 0.562   | 4.48    | 4.36     | 0.173    |
|           | +    | 0.037  | 332       | 0.566   | 1.54    | 0.434   | 5.72    | 4.64     | 0.219    |
| W-466     | −    | 0.113  | 330       | 0.455   | 1.03    | 0.545   | 4.69    | 4.12     | 0.095    |
|           | +    | 0.069  | 330       | 0.415   | 1.07    | 0.585   | 4.46    | 3.96     | 0.129    |
| W-558     | −    | 0.055  | 335       | 0.476   | 1.05    | 0.524   | 4.71    | 4.09     | 0.105    |
|           | +    | 0.028  | 335       | 0.573   | 1.32    | 0.427   | 5.29    | 4.29     | 0.116    |
| NATA      | −    | 0.14   | 349       | 1.0     | 2.93    |         |         | 2.93     | ND       |
|           | +    | 0.14   | 349       | 1.0     | 2.83    |         |         | 2.83     | ND       |

### Notes
- The excitation wavelength was 295 nm and the emission was scanned from 305 to 450 nm with a 4-nm bandpass for both. Protein concentration was adjusted in the protein samples so that the A295 was between 0.05 and 1. The proteins were in 100 mM NaCl, 20 mM Tris-HCl buffer, pH 7.4, and 25 °C with NATA (Qe = 0.14) as the quantum standard.
- Steady state fluorescence emission maxima (corrected for λ-dependent bias of the emission monochromator and detector system); S.E. ± 1 nm.
- Normalized pre-exponential factor obtained from the fit of the time-resolved decay data.
- Fluorescence decay times recovered from the fit to the sum of exponentials: R(t, λ) = Σαiλiexp(−t/τi), where τi is the decay time of the ith component and qi(λ) is the pre-exponential factor at the emission wavelength λ.
- The individual fluorescence decay times, τi, and the normalized pre-exponential values, αi, were used to calculate the τave values (intensity-weighted average lifetimes), where \( τ_\text{ave} = Σαiτi^2/Σαi. \)
- The steady-state anisotropy values (rave) were determined under conditions as described above with the excitation at 295 nm and emission at 340 nm.
- Errors are the S.D. of at least three independent experiments and are shown in parentheses for Qe and rave values.
- Not determined.


**TABLE II**

NAD$^+$ binding and GH activity of PE24 wild-type and Trp mutants

| Protein | $K_h^{a}$ | Quenching$^{b}$ | GH activity$^{c}$ |
|---------|-----------|----------------|------------------|
| Wild-type | $47 (2)^d$ | $73 (16)^d$ | $100 ^d$ |
| W-417 | $55 (12)$ | $63 (2)$ | $87 (12)$ |
| W-466 | $63 (20)$ | $39 (7)$ | $73 (15)$ |
| W-558 | $296 (47)$ | $49 (11)$ | $25 (5)$ |
| Trp | | | $16 (3)$ |

$^{a}$ Determined from Scatchard analysis of the percentage of fluorescence quenching as a function of NAD$^+$ concentration; values represent the mean ± S.D. from three independent experiments.

$^{b}$ Estimated from the maximum level of fluorescence quenching that occurred as described in the table legend and that can be seen in Fig. 3. GH activity for the WT protein at 400 μM x-NAD$^+$ showed the following kinetic parameters: $V_{\text{max}} = 3.34 ± 1.00 \mu \text{mol/liter} \cdot \text{h}$, $K_m = 49.2 ± 7.8 \mu \text{M}$; and $k_{\text{cat}} = 1.67 ± 0.5 \text{h}^{-1}$. Errors are the S.D. of at least three independent experiments and are shown in parentheses.

$V_{\text{max}} = 3.34 ± 1.00 \mu \text{mol/liter} \cdot \text{h}$, $K_m = 49.2 ± 7.8 \mu \text{M}$; $k_{\text{cat}} = 1.67 ± 0.5 \text{h}^{-1}$; and specificity constant, 0.0338 μmol/liter-h. Both W-417 and W-466 mutant proteins possessed the same activity as the WT protein, but the W-558 and Trp-deficient mutant proteins were slightly less active. These results compare favorably to an earlier report involving n-1 and n-2 Trp mutant proteins of a larger catalytically active fragment of ETA, PE40 (9).

**Fluorescence Anisotropy and NAD$^+$ Binding**—The effect of NAD$^+$ substrate binding to the PE24 active site was also monitored by fluorescence anisotropy determinations (Table I). The WT protein possessed a moderate anisotropy value in the absence of bound substrate (0.118), which showed a significant increase upon association with NAD$^+$ (0.207, Table I). The single Trp mutant proteins showed a range of $r_{\text{ave}}$ values in the absence of bound substrate, ranging from 0.095 (W-466) to 0.173 (W-417). W-417 showed the largest increase in its $r_{\text{ave}}$ value upon binding the NAD$^+$ substrate ($\Delta r_{\text{ave}} = 0.046$), followed in decreasing order by W-466 ($\Delta r_{\text{ave}} = 0.034$) and finally by W-558 ($\Delta r_{\text{ave}} = 0.005$). These data indicate that the largest structural change within the catalytic domain upon NAD$^+$ substrate binding occurs in the vicinity of Trp-417.

**Stern-Volmer Quenching**—The Stern-Volmer quenching profiles are shown in Fig. 4. The WT protein showed a protection of the Trp fluorescence quenching induced by acrylamide in the presence to NAD$^+$. This protection was also observed for the W-558 mutant protein, but there was no significant difference for either the W-417 or W-466 mutant proteins. The quenching profiles were linear and were best fit by correlation coefficients between 0.981 and 0.999. The Stern-Volmer constants for these proteins are shown in Table III. In the absence of substrate, the WT showed the highest value (8.28 M$^{-1}$), followed in decreasing order by W-417, W-558, and W-466. The bimolecular quenching constants ($k_q$) are also shown in Table III. The two single Trp mutant proteins, W-417 and W-466, exhibited the highest collisional constants (1.77 and 1.80 M$^{-1}$ns$^{-1}$, respectively), followed by the WT protein (1.41 M$^{-1}$ns$^{-1}$) and finally by W-466 (1.32 M$^{-1}$ns$^{-1}$). NATA was included as a control and under the same conditions as for the proteins showed a value greater than 11 M$^{-1}$ns$^{-1}$ (Table III). Upon binding the NAD$^+$ substrate the value for the Stern-Volmer constant decreased for the WT protein (2.89 M$^{-1}$). The collisional constant also showed a significant decrease from 1.41 to 1.04 M$^{-1}$ns$^{-1}$. The W-417 protein showed a small decrease in solvent exposure of Trp-417 upon substrate binding (1.77 to 1.62 M$^{-1}$ns$^{-1}$) in contrast to W-466, which showed an increase in the solvent exposure of its Trp residue (1.32 to 1.58 M$^{-1}$ns$^{-1}$). The W-558 mutant protein showed a significant decrease in both the Stern-Volmer constant (7.35 to 4.70 M$^{-1}$) and the collision constant (1.80 to 1.12 M$^{-1}$ns$^{-1}$). This indicates that the substrate docks near Trp-558 and provides some solvent shielding for this residue. This effect seems to be intensified within the WT protein, which may reflect some local perturbation of the active site caused by the Trp → Phe substitutions.

**Structural Integrity and Folding of PE24**—The titration profiles for the denaturation of WT PE24 with urea and Gn-HCl are shown in Fig. 5, A and B, respectively. The α-helical content of the WT PE24 protein was determined by CD spectroscopy to be 19%. Importantly, there was no significant changes in the average secondary structure content for any of the single Trp mutant proteins (data not shown). The unfolding of PE24 was reversible in both types of denaturants (Fig. 5, A and B). The calculated $\Delta G_U$ values for the WT protein was dependent upon the method used to measure secondary structure changes associated with denaturation. Values of 10.7 ± 1.7 and 13.8 ± 3.8 kJ/mol for the $\Delta G_U$ (urea denaturant) were obtained for the fluorescence-based and the CD-based measurements, respectively (Table IV). The difference was even more pronounced between the $\Delta G_U$ values for fluorescence and CD measurements in Gn-HCl (8.7 ± 2.9 and 19.6 ± 3.3 kJ/mol, respectively). The $\Delta G_U$ values for all the mutant proteins were identical to the WT protein as assessed by Gn-HCl denaturation experiments using fluorescence spectroscopy (8.7 ± 1.1). Interestingly, when NAD$^+$ was bound to WT PE24 at saturating concentration, the enzyme was stabilized by 7.9 kJ/mol (Table IV). The rates for the unfolding/folding processes were determined by stopped flow fluorescence spectroscopy, and a sample trace is shown in Fig. 5C. The rate constants for the unfolding reaction were much slower than for the folding reaction (Table IV). All the rate processes were faster in the Gn-HCl denaturant as compared with urea, and the $k_f$ constant was too rapid for determination by conventional stopped flow instrumentation (instrumental dead-time = 1.3 ms).

**DISCUSSION**

The quantum yield of the WT PE24 protein is not the average of that measured for the three single Trp mutant proteins (WT, 0.17; mutant average, 0.09). This indicates that there must be some degree of local perturbation of the protein environment upon Trp → Phe substitution within PE24 or energy transfer between Trp residues of the WT. However, the weighted average for the anisotropy of the three single Trp mutant proteins is similar to the measured value for the WT anisotropy, indicating little or no depolarization of the WT anisotropy value. This effectively rules out the latter possibility. The origin of the low quantum yield for Trp-558 is likely its close position to the guanido side chain of Arg-467 in addition to its high degree of aqueous solvent exposure. It is well known that Arg will quench indole fluorescence, although the mechanism is not well understood (21). All three Trp residues showed a significant degree of fluorescence quenching upon NAD$^+$ binding. In the case of Trp-558, because the adenine ring of NAD$^+$ docks next to the benzene portion of the indole, a ground state nucleic acid-indole complex could be formed (9, 22). However, the origin of the fluorescence quenching of Trp-417 and Trp-466 is less obvious. Previously it was suggested that a conserved loop region within ETA (residues 456–470, and residues 39–46 in DT) that harbors two Asp residues, Asp-461 and Asp-463, could be the source of substrate-induced fluorescence quenching within the active site of the toxin (9). Unfortunately, this loop is unresolved in the x-ray structure of the PE24-β-TAD substrate complex (10). The largest change in the fluorescence quantum yield upon substrate binding was observed for Trp-417. Given that both bases of the NAD$^+$ substrate are no closer than 17 Å to the Trp-417 indole ring, the origin of the fluorescence...
cence quenching mechanism of this Trp must lie in a structural change within the protein segment surrounding it. A comparison of the protein environment of Trp-417 in the substrate bound and substrate-free forms revealed that the likely candidates for the fluorescence quenching are a number of peptide bonds from various residues including Thr-418 (23).

Upon NAD$^+$ binding, the anisotropy of the WT increased (Table I), and this increase was reflected in the anisotropies of the both single Trp mutant proteins, W-417 and W-466, the former showing the greatest change. This adds further credence to the idea that there is a substrate-induced structural change within the protein near Trp-417. Trp-558 was previously believe to dock against the adenine ring of NAD$^+$ (9). However, with the advent of the $\beta$-TAD$^+$-PE24 structure (10), it can be seen that there is 3–4 Å between the Trp-558 and $\beta$-TAD$^+$ rings. The anisotropy results for W-558 binding to NAD$^+$ confirm this observation because the Trp-558 $r_{ave}$ value does not change significantly upon substrate binding (Table I). If there was direct van der Waals contact between the two ring structures, an increase in Trp-558 anisotropy would be expected.

The $k_q$ value for the WT protein was similar to the weighted average values for the three single Trp mutant proteins (1.41 and 1.58 M$^{-1}$ ns$^{-1}$, respectively). Trp-417 and Trp-558 were similar in their extent of solvent accessibility, and both were considerably more exposed to solvent than was Trp-466. These findings were consistent with earlier suggestions based on x-ray data (10) and sensitivity to NBS oxidation (9). Trp-417...
**TABLE IV**  
Parameters for unfolding/refolding of wild-type PE24

| Parameters determined from the data shown in Fig. 5. | Urea | Gn-HCl |
|---|---|---|
| Denaturant | ku | t1/2 | Ku | ΔG_u/F_NAD | ΔG_u/F | ΔG_u/CD |
| Urea | 0.25 (0.2) \( s^{-1} \) | 496 (10) \( s^{-1} \) | 1.4 | 5.0 \( \times 10^{-4} \) | 10.7 (1.7) \( kJ/mol \) | 13.8 (3.8) \( kJ/mol \) |
| Gn-HCl | 23.6 (2.0) \( kJ/mol \) | 73750 \( kJ/mol \) | 0.009 | 3.2 \( \times 10^{-4} \) | 16.5 (5.8) | 8.7 (2.9) |

**a** Rate constant for the unfolding reaction for PE24.  
**b** Rate constant for the folding reaction for PE24.  
**c** Reaction half-life calculated based on a pseudo first order reaction where \( t_{1/2} = \ln 2/k \).  
**d** Equilibrium constant for the reaction: \( \text{PE24}_{\text{native}} \rightleftharpoons \text{PE24}_{\text{denatured}}, \) where \( K_u = [\text{PE24}_{\text{denatured}}]/[\text{PE24}_{\text{native}}]. \)  
**e** Gibbs free energy for the unfolding reaction in the presence of 500 \( \mu M \) NAD\(^+\) where \( \Delta G_u = -RT\ln K_u \) as determined by Trp fluorescence spectroscopy.  
**f** Gibbs free energy for the unfolding reaction as determined by Trp fluorescence spectroscopy in the absence of NAD\(^+\).  
**g** Gibbs free energy for the unfolding reaction (no NAD\(^+\)) as in Footnote \( f \) but determined by circular dichroism spectroscopy.  
**h** Errors are the S.D. of three or four independent experiments and are shown in parentheses.  
**i** Rate constant was calculated based on the \( \Delta G_u \) value obtained by CD analysis (no NAD\(^+\)).

**Fig. 5.** Unfolding, refolding, and stopped flow profiles for WT PE24. The conditions for the experiments were as described under “Experimental Procedures.” The samples include unfolding (●) and refolding (○) profiles in urea (A), unfolding (●) and refolding (○) profiles in Gn-HCl (B), and stopped flow kinetic trace for the unfolding process (0–4 M Gn-HCl) (C).

| Parameters determined from the data shown in Fig. 5. | Urea | Gn-HCl |
|---|---|---|
| Denaturant | ku | t1/2 | Ku | ΔG_u/F_NAD | ΔG_u/F | ΔG_u/CD |
| Urea | 0.25 (0.2) \( s^{-1} \) | 496 (10) \( s^{-1} \) | 1.4 | 5.0 \( \times 10^{-4} \) | 10.7 (1.7) \( kJ/mol \) | 13.8 (3.8) \( kJ/mol \) |
| Gn-HCl | 23.6 (2.0) \( kJ/mol \) | 73750 \( kJ/mol \) | 0.009 | 3.2 \( \times 10^{-4} \) | 16.5 (5.8) | 8.7 (2.9) |

**a** Quenching experiments were conducted in the presence (+) or absence (−) of 500 \( \mu M \) NAD\(^+\).  
**b** \( \tau_{ave} \) represents the intensity-weighted fluorescence lifetime as described in Table I.  
**c** The Stern-Volmer quenching constants were obtained from the slopes of the lines for the plots of \( F_0/F = 1 + K_{SV}[Q]. \)  
**d** \( k_q \) is the bimolecular quenching constant for the acrylamide:Trp (quencher/fluorophore) pair. This constant is determined from the relationship \( K_{SV} = k_t/k_q. \)  
**f** Errors are the S.D. of three or four independent experiments and are shown in parentheses.
remains solvent exposed upon substrate binding, whereas Trp-466 becomes more exposed. In contrast, Trp-558 is protected from solvent upon substrate complexation with the enzyme. This latter observation is likely due to the close proximity of NAD$^+$ to the Trp-558 residue.

The GH activity of the various single Trp mutant proteins mirrored our earlier report that none of the Trp residues were critical for GH activity of the toxin-enzyme (9). The measured turnover number ($k_{cat}$ value) was also nearly identical to the reported value for WT PE40 (1.67 ± 0.5 and 1.84 ± 0.4 h$^{-1}$ for PE24 and PE40, respectively) previously reported by our laboratory. The GH activity was the lowest for the triple mutant PE24 and PE40, respectively) for the NAD$^+$ substrate (Table IV). Remarkably, the stability of WT PE24 protein was increased by 2-fold upon complexation of the NAD$^+$ substrate (Table IV). This may indicate that when both Trp-417 and Trp-466 are replaced by Phe, there is a cumulative structural change within the active site of the protein that leads to a less effective binding pocket. The replacement of Trp-558 by Phe does not seem to have any such effect (Table II).

The enzyme domain of ETA alone (domain III) is less stable than the whole toxin or PE40 (domains II and III) (Table IV) (24). Remarkably, the stability of WT PE24 protein was increased by 2-fold upon complexation of the NAD$^+$ substrate (Table IV). The stopped flow experiments using denaturants as unfolding agents revealed that PE24 (domain III) refolds extremely quickly—much more rapidly than either whole toxin or PE40. This was proposed from our earlier folding experiments involving whole toxin and PE40 (24). The biological implications of the rapid refolding process of domain III may reflect the need for this domain to refold upon translocation to the cytoplasm upon crossing the endoplasmic reticulum membrane in order to avoid destruction by host endogeneous proteinases or other scavenging cellular machinery during the intoxication process.

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