Insight into the Catalytic Mechanism of Pseudomonas aeruginosa Exotoxin A

STUDIES OF TOXIN INTERACTION WITH EUKARYOTIC ELONGATION FACTOR-2*

Received for publication, July 10, 2002, and in revised form, August 19, 2002 Published, JBC Papers in Press, September 20, 2002 DOI 10.1074/jbc.M206916200

The molecular nature of the protein-protein interactions between the catalytic domain from Pseudomonas aeruginosa exotoxin A (PE24H) and its protein substrate, eukaryotic elongation factor-2 (eEF-2) were probed using a fluorescence resonance energy transfer method. Single cysteine mutant proteins of PE24H were prepared and site-specifically labeled with the donor fluorophore IAEDANS (5-(2-iodoacetylaminoethyl)-1-naphthalenesulfonic acid), whereas eEF-2 was labeled with the acceptor fluorophore fluorescein. The association was found to be independent of ionic strength and of the co-substrate, NAD⁺ but dependent upon pH. The lack of requirement for NAD⁺ to produce the toxin-eEF-2 complex demonstrates that the catalytic process is a random order mechanism, thereby disputing the current model. The previously observed pH dependence for catalytic function can be assigned to the toxin-eEF-2 binding event, as the pH dependence of binding observed in this study showed a strong correlation with enzymatic activity. The ability of the toxin to bind eEF-2 with bound GTP/GDP was assessed using nonhydrolyzable analogues. The results from the substrate binding and catalytic activity experiments indicate that PE24H is able to interact and bind with eEF-2 in all of its guanyl nucleotide-induced conformational states. Thus, the toxin ribosylates eEF-2 regardless of the nucleotide-charged state of eEF-2. These results represent the first detailed characterization of the molecular details and physiological conditions governing this protein-protein interaction.

The important catalytic residues for ETA are Glu-553, His-440, located at the base of the active site cleft, hydrogen bonds with the AMP ribose moiety from NAD⁺ to eEF-2, and ultimately leading to cellular death (5, 8).

The three-dimensional structure of ETA that shows it consists of three distinct functional domains: receptor-binding (domain I), translocation (domain II), and catalysis (domain III) (9, 10). The catalytic domain (residues 400–613) is responsible for the inactivation of eEF-2 by catalyzing the transfer of the ADP-ribose moiety from NAD⁺ to eEF-2. It is proposed that initially a binary complex forms between NAD⁺ and the catalytic domain followed by the subsequent binding of eEF-2, representative of an ordered sequential reaction (11, 12). In addition, stereochemical and kinetic data suggest that the reaction mechanism is likely an S₅₋₁ nucleophilic substitution involving an oxocarbonium cation, despite the observed inversion of configuration for the glycosidic bond formed between the ribose and diphthamide (5, 11, 13, 14). This inversion most likely occurs because the anomic carbon of the nicotinamide ribose is susceptible to a backside nucleophilic attack from the N-1 of the imidazole portion of diphthamide (5).

The important catalytic residues for ETA are Glu-553, His-440, Tyr-481, and Tyr-470, which were identified by the important catalytic residues for ETA are Glu-553, His-440, Tyr-481, and Tyr-470, which were identified by structural and mutagenesis studies (9, 13, 34, 35). The structure of the catalytic domain shows that the imidazole side chain of His-440, located at the base of the active site cleft, hydrogen bonds with the AMP ribose moiety of NAD⁺ and with the main chain carbonyl of Tyr-470 (13). The nicotinamide ring stacks with Tyr-481 and Tyr-470 and is located near the nicotinamide where it is involved in Van der Waals interactions with the thiazole-ribose moiety of the nonhydrolyzable NAD⁺ analogue, β-TAD. Glu-553 forms a hydrogen bond with the oxygen of the amino-1-naphthalenesulfonic acid: eEF-2, eukaryotic elongation factor-2; eEF-2-AF, 5-AF labeled eEF-2; FRET, fluorescence resonance energy transfer; GDP-β-S, guanosine 5′-O-(2-thiodiphosphate); GDP-γ-S, guanosine 5′-O-(3-thiotriphosphate); Kᵦ, substrate binding (association) constant; Kᵦᵦ, substrate binding (dissociation) constant; o-GTP, oxidized GTP; PE24H, Pseudomonas aeruginosa exotoxin A, 24-kDa C-terminal fragment containing a hexa-His tag.

This paper is available on line at http://www.jbc.org
duced using GTP and GDP nonhydrolyzable analogues. eEF-2 is a single polypeptide chain with a molecular mass ranging from 90 to 110 kDa depending on the source of its isolation (15). eEF-2 is a factor involved in the elongation phase of protein synthesis and is a member of the GTPase superfamily. Specifically, eEF-2 is responsible for catalyzing the translocation of the peptidyl-tRNA from the A-site to the P-site within the ribosome, movement of the deacylated tRNA from the P-site to the E-site, and finally movement of the mRNA to allow the next codon to be placed in the A-site (16). eEF-2 contains a post-translationally modified histidine residue, 2-[3-carboxamido-3-(trimethylammonio)propyl]histidine, referred to as diphthamide (17, 18). This residue is located at position 715 in the rat liver eEF-2 sequence near the C terminus of the protein (19). Diphthamide has only been found in eEF-2 and is completely conserved throughout all eukaryotic and archaeabacterial evolution (18, 20). Although the diphthamide is the site of toxin attack, it does not appear to be necessary for normal eEF-2 function.

Currently, no three-dimensional structure has been reported for eEF-2, which may help to explain the lack of understanding regarding the toxin-eEF-2 interaction. The aim of the present study was to investigate the physiological conditions requisite for the interaction between eEF-2 and the catalytic domain of ETA using a fluorescence resonance energy transfer (FRET) approach. We introduced single cysteine residues into the catalytic domain of ETA and labeled the domain with a donor fluorophore, whereas the protein substrate, eEF-2 was labeled with the acceptor fluorophore. The integrity of the labeled toxins was assessed through enzyme activity and substrate binding measurements. Furthermore, the dissociation constant for eEF-2 binding to the toxin was also determined. This binding event was shown to be pH-dependent but insensitive to the ionic strength of the aqueous environment and to the presence of the substrate, NAD+. Finally, the importance of the conformation of eEF-2 on toxin ADP-ribosylation activity was evaluated using GTP and GDP nonhydrolyzable analogues.

**EXPERIMENTAL PROCEDURES**

**Overexpression and Purification of PE24H**—The catalytic fragment of ETA with a C-terminal 6-His tag (PE24H) was overexpressed and purified as described (21) except that all cysteine-containing mutant proteins required the chelate-agarose affinity column to be charged with 100 mM ZnSO4.

**Purification of eEF-2**—eEF-2 was purified from wheat germ as previously described (21) with some modifications.

**Site-directed Mutagenesis**—Single cysteine mutant proteins were created using either Kunkel mutagenesis (22) or by standard QuickChange™ mutagenesis (Stratagene, La Jolla, CA). The DNA template was the plasmid pH1 containing the gene for wild-type PE24H. The desired mutation, determined by dideoxy sequencing with an ABI Prism Model 377 DNA sequencer using dye termination and cycle sequencing, was analyzed on a 4.5% acrylamide gel.

**Fluorescence Labeling of PE24H**—The PE24H protein was labeled with the fluorophore IAEDANS as detailed earlier (8) with some modifications. The labeling efficiency, based on the appropriate absorbance and molar extinction coefficients of the bound fluorophore and the measured protein, ranged between 80 and 100%, with the exception of S408C, which was only 60%.

**Fluorescence Labeling of eEF-2**—Purified wheat germ eEF-2 protein was labeled with 5-indocarbamoyl fluorescein as described previously (8) with some modifications.

**Fluorescence and Spectroscopic Measurements**—All steady-state fluorescence measurements were obtained using a PTI Alphascan spectrophotometer interfaced with a computer using Felix™ Version 1.21 software (Photon Technology International, South Brunswick, NJ) and equipped with a water-jacketed sample chamber set to 25 °C. The excursions were the determination of the pH profile for eEF-2 binding and the effects of guanyl nucleotides on binding, for which we used a Cary Eclipse fluorometer (Varian, Mississauga, Ontario, Canada) equipped with the feldt Biotest thermostatted multicell holder at 25 °C. The PE24H-AEDANS excitation (λex = 480 nm) and emission spectra (λem = 515 nm) and emission spectra (λem = 492 nm) were obtained for proteins in 100 mM KCl, 20 mM Tris-HCl (pH 7.9) buffer. The eEF-2-AF excitation (λem = 515 nm) and emission spectra (λem = 492 nm) were recorded for proteins in 50 mM NaCl, 20 mM Tris-HCl (pH 7.9) buffer. Absorbance spectra were obtained at room temperature with a PerkinElmer Lambda-4 double beam, scanning absorption spectrometer (PerkinElmer Life Sciences) interfaced to a personal computer. PE24H-ADP-ribosylation assays were scanned from 200 nm to 600 nm in 50 mM NaCl, 20 mM Tris-HCl (pH 7.9) solution. eEF-2-AF absorbance spectra were recorded from 250 to 600 nm in 300 mM KCl, 20 mM Tris-HCl (pH 7.9) buffer.

**Fluorescence-based ADPRT Assay**—The NAD+-dependent ADPRT activity for the various cysteine PE24H proteins and their corresponding protein adducts was determined as described by Armstrong and Merrill (21).

The effect of GTP- and GDP-bound eEF-2 was assessed with the substrates NAD+ (500 μM) GTP-γ-S, and eEF-2 (14 μM; with or without bound guanyl nucleotides) held at saturating levels. The assay was as described above except that the nucleotides (GDP-β-S at 50 μM, Sigma) were initially allowed to bind to eEF-2 for 10 min at 25 °C and the reaction buffer was supplemented with 8 mM MgCl2.

**Quenching of Intrinsic Protein Fluorescence**—The NAD+-dependent quenching of the intrinsic tryptophan fluorescence in PE24H was used to determine the binding constant (Kd) for NAD+ as described elsewhere (5).

**Fluorescence-based eEF-2 Binding Assay**—The FRET-based assay was conducted as described previously (8). The dissociation constant for eEF-2 binding with PE24H was determined using the following equation as part of the nonlinear fitting function of Origin 6.1 (OriginLab, Northampton, MA): ΔF/ΔFmax = (Kd + [eEF-2])/([eEF-2] + Kd), where ΔF is the change in fluorescence intensity for each ligand (e.g. a macromolecular association), ΔFmax is the maximum change in fluorescence intensity at saturation of the ligand-binding site within PE24H, Kd is the dissociation constant for the binding of eEF-2 with PE24H, and Bmax is the total PE24H concentration (number of binding sites, as there is 1 site/protein).

S585C-AEDANS was chosen as the control protein to evaluate the effects of NAD+ urea, salt, pH, and guanyl nucleotides on the intrinsic eEF-2 binding. S585C and its adduct behave similarly to the wild-type enzyme in terms of its catalytic activity and NAD+ binding; also this protein labeled with IAEDANS at a high efficiency.

The requirement of NAD+ for protein complexation was evaluated using the nonhydrolyzable analogue, β-TAD, at a concentration of 200 μM. The ability of denatured PE24H-AEDANS to bind eEF-2-AF was evaluated in buffer containing 4 mM urea (Pierce), which denatures the PE24H protein but does not unfold the eEF-2 protein.

The effect of various anions on eEF-2 binding to PE24H was measured by varying the salt concentration from 0 to 800 mM in the binding buffer. The Tris-HCl buffer (20 mM, pH 7.9) was supplemented with KCl, Na2SO4, or NaF.

The effect of pH on the binding was obtained by varying the pH between 4 and 12. The buffer contained 50 mM NaCl, and the buffering components (pH range shown in parentheses) were sodium acetate (30 mM, pH 4), bis-Tris (30 mM, pH 6.5–6.8), Tris-HCl (30 mM, pH 7–8.8), and CAPS (30 mM, pH 9–12). The Kd values were converted to Ks values for plotting purposes using the expression, Ks = 1/Kd. The effects of guanyl nucleotides bound to eEF-2-AF was assessed in 50 mM NaCl, 20 mM Tris-HCl (pH 7.9) supplemented with 8 mM MgCl2 with either 50 μM GTP-γ-S or GDP-β-S (Sigma). eEF-2-AF was preincubated with the nonhydrolyzable guanyl nucleotides (at 50 μM) for 10 min before titration with the toxin. The saturating concentration of these nucleotides is 2-3 μM (23).

**RESULTS**

**Characterization of the Single Cysteine Mutant Proteins and Their Derivatives**—Intrinsic fluorescence-based techniques for studying the interactions between PE24H and eEF-2 have proved difficult because both proteins possess tryptophan fluorescence. The catalytic domain of ETA, PE24H, does not contain any native cysteine residues. Therefore, single cysteine residues were introduced into the protein at defined sites.
within the toxin. The sites chosen for mutagenesis included those that surround the NAD⁺-binding site as well as surface-exposed sites distant from the active site (Fig. 1). All mutant proteins were used to assess the interactions between the toxin and eEF-2. The thiol-specific reaction between the cysteine residues within PE24H and the fluorophore, IAEDANS, created proteins that contained a single, covalently linked reporter fluorophore. AEDANS was chosen as the fluorophore because of its large Stokes shift and because it has an emission spectrum that overlaps well with the absorbance of other fluorescent dyes such as fluorescein. AEDANS is ideal for FRET experiments of up to ~60 Å (24).

The structural integrity of the mutant PE24H proteins with and without the attached fluorophore was assessed by both NAD⁺ substrate binding and ADPRT enzymatic activity. None of the single cysteine replacements in PE24H led to significant changes in either the affinity for the NAD⁺ substrate or in ADPRT activity (Table I). Generally, introduction of the fluorophore also did not cause major perturbations of the proteins as reflected by measurements of the transferase and binding activities of the mutant adduct proteins (Table II). However, T442C, S449C, and E486C-AEDANS showed reduced ADPRT activity upon alkylation (84-, 16-, and 12-fold decrease, respectively) (Table II). Thr-442, situated on the same β-strand as His-440, and Ser-449, located in an α-helix near the opening of the active site cleft, both hydrogen bond with NAD⁺ (11, 13) (Fig. 1) and furthermore, Glu-486 is present in the loop region, which was previously identified as modulating the transferase activity of the toxin (8) (Fig. 1). Therefore, the decreased activity of these three mutant proteins is likely a reflection of the spatial importance of these residues for catalytic function; when the AEDANS fluorophore is present at these sites within the corresponding protein adducts, it may sterically interfere with normal enzyme function.

A typical UV-visible absorption spectrum for PE24H-AEDANS is shown in Fig. 2A. It is characterized by maxima at 260, 285, and 337 nm; the latter peak was used to determine the labeling stoichiometry of the various PE24H-AEDANS adducts. Also, the fluorescence spectra for PE24H-AEDANS (Fig. 3A) show excitation and emission maxima for PE24H-AEDANS at 337 and 480 nm, respectively.

**Table I**

Comparison of the ADPRT activity and NAD⁺-binding affinity for the cysteine replacement mutants

| Protein      | \(k_{cat}\)  | \(K_{NAD⁺}\)  |
|--------------|-------------|---------------|
| Wild type    | 675 ± 85    | 55 ± 6        |
| S405C        | 630 ± 108   | 59 ± 3        |
| S410C        | 184 ± 31    | 50 ± 6        |
| T442C        | 132 ± 33    | 35 ± 3        |
| S449C        | 234 ± 42    | 129 ± 7       |
| S459C        | 310 ± 113   | 55 ± 10       |
| E486C        | 930 ± 127   | 57 ± 8        |
| S507C        | 1256 ± 61   | 63 ± 2        |
| S515C        | 307 ± 76    | 48 ± 5        |
| S558C        | 731 ± 145   | 61 ± 4        |

\(\text{Not applicable, as wild-type PE24H contains no endogenous cysteine and therefore cannot be labeled and studied in the FRET-based eEF-2 binding assay. However, an independent eEF-2 binding assay involving the incorporation of 5-hydroxytryptophan into the wild-type PE24H protein gave a } K_d \text{ for eEF-2 of 0.98 ± 0.06 } \mu\text{M (27).}\)

**Table II**

Comparison of the ADPRT activity, NAD⁺, and eEF-2 binding for the cysteine replacement mutants labeled with the fluorophore IAEDANS

| Protein      | \(k_{cat}\)  | \(K_{NAD⁺}\)  | \(K_{eEF-2}\)  |
|--------------|-------------|---------------|---------------|
| Wild type    | 675 ± 85    | 55 ± 6        | NA*           |
| S405C-AEDANS | 435 ± 43    | 44 ± 10       | 1.2 ± 0.3     |
| S410C-AEDANS | 367 ± 65    | 51 ± 11       | 0.9 ± 0.1     |
| T442C-AEDANS | 8 ± 1       | 5300 ± 300    | 1.2 ± 0.1     |
| S449C-AEDANS | 43 ± 7      | 546 ± 14      | 0.9 ± 0.1     |
| S459C-AEDANS | 542 ± 66    | 36 ± 4        | 0.9 ± 0.1     |
| E486C-AEDANS | 56 ± 5      | 115 ± 4       | 0.8 ± 0.2     |
| S507C-AEDANS | 268 ± 50    | 30 ± 4        | 1.1 ± 0.2     |
| S515C-AEDANS | 396 ± 73    | 87 ± 15       | 1.0 ± 0.1     |
| S558C-AEDANS | 192 ± 61    | 30 ± 3        | 1.9 ± 0.2     |

*Not applicable, as wild-type PE24H contains no endogenous cysteine and therefore cannot be labeled and studied in the FRET-based eEF-2 binding assay. However, an independent eEF-2 binding assay involving the incorporation of 5-hydroxytryptophan into the wild-type PE24H protein gave a \(K_d\) for eEF-2 of 0.98 ± 0.06 \(\mu\)M (27).
assessed when PE24H-AEDANS was chemically denatured with urea (Fig. 3B). PE24H-AEDANS is completely unfolded in 4 M urea (25); however, eEF-2-AF, a more structurally stable protein, remains in its native folded state at this denaturant concentration (data not shown). As shown by the data in Fig. 3B, no FRET occurs between denatured PE24H-AEDANS and folded eEF-2-AF, thereby providing evidence that the FRET assay is a good measure of the specific nature for the association between the toxin and eEF-2.

The inset to Fig. 3B shows a decrease in the fluorescence intensity at 460 nm as the concentration ratio of eEF-2-AF and PE24H-AEDANS approaches unity with no further change once the stoichiometry is about 2.5:1 (eEF-2:PE24H). These findings are indicative of a specific interaction between the two proteins characterized by tight binding. Binding constants for this protein-protein interaction were determined (Table II) and ranged from 0.8 to 1.9 μM for the various labeled mutant proteins.

**Effect of NAD⁺ on eEF-2 Binding to the Toxin**—Using this FRET-based assay, the requirement of NAD⁺ for the binding of eEF-2 to PE24H was also assessed. To form a stable PE24H-eEF-2-NAD⁺ complex that is incapable of completing the transferase reaction, the nonhydrolyzable analogue of NAD⁺, β-TAD, was used. The binding of eEF-2 to PE24H was examined both in the absence and presence of β-TAD. The similarity in the binding isotherms (Fig. 3B) was indicative that PE24H does not require NAD⁺ in order to associate with eEF-2. The eEF-2 binding constants were determined to be 2.7 (± 0.3) and 1.9 (± 0.2) μM, respectively, for the presence and absence of β-TAD.

**Interaction of PE24H with eEF-2**—The effect of KCl concentration on the interaction between PE24H and eEF-2 was also investigated. Surprisingly, eEF-2 binding to PE24H was not significantly influenced by increases in KCl concentration (Fig. 4A), with the $K_D$ varying only slightly, from 2.0 to 1.3 μM, for S585C-AEDANS binding with the translocase protein, which represents a 1.5-fold increase in affinity. Similarly, Na₂SO₄ and NaF had no influence on the association of these proteins (data not shown). Therefore, the protein-protein interaction that is such an integral part of the catalytic mechanism of the PE24H enzyme is not sensitive to the ionic strength of the solution.

The pH profile for eEF-2 binding to PE24H (Fig. 4B) shows a typical bell-shaped curve, which was fit to a Gaussian function. Notably, the pH dependence of this association could not be determined close to the pI for eEF-2 (5.0–5.3) due to its pre-

---

*Fig. 2. Absorbance and fluorescence spectra of PE24H-AEDANS and eEF-2-AF.* The absorbance spectra of PE24H-AEDANS (A) and eEF-2-AF (B) were normalized at 280 nm. The fluorescence excitation (solid line) and emission spectra (dashed line) of PE24H-AEDANS (6.0 μM; λₑₐₓ = 480 nm and λₑₐₘ = 337 nm for the excitation and emission spectra, respectively) (C) and eEF-2-AF (3.5 μM; λₑₐₓ = 515 nm and λₑₐₘ = 492 nm for the excitation and emission spectra, respectively) (D) were normalized to the corresponding peak maximum values. The emission and excitation slits were both set to 2 nm. Other conditions were as described under “Experimental Procedures.”
cipitation, as previously observed when assessing the pH dependence for catalytic function (21). The pH optimum for eEF-2 binding to the toxin is at 7.8 and has two $pK_a$ values. The acidic and alkaline $pK_a$ values are 6.3 and 9.3, respectively. Moreover, the binding affinity was negligible at pH values less than 4 and greater than 10.

In fluorescence intensity of eEF-2 indicated an association of these nucleotides with eEF-2 at the saturating concentration of 50 $\mu M$, which is in agreement with the earlier findings of Sontag et al. (23). As Fig. 5 illustrates, the toxin bound eEF-2-AF regardless of whether GTP-$\gamma$-S or GDP-$\beta$-S was bound to the protein substrate. The binding constants for eEF-2-AF, eEF-2-AF-GTP-$\gamma$-S, and eEF-2-AF-GDP-$\beta$-S with PE24H-AEDANS were determined to be 2.0 ($\pm$ 0.3), 1.6 ($\pm$ 0.2), and 2.1 ($\pm$ 0.3) $M^{-1}$, respectively. In addition, when eEF-2 was preincubated with either GTP-$\gamma$-S or GDP-$\beta$-S, the ADPRT activity of the toxin remained constant, indicating that eEF-2 in the absence or presence of bound guanyl nucleotides can still serve as a substrate for PE24H (Table III).

DISCUSSION

All cysteine-labeled PE24H mutants in this study generally behaved similarly to the wild-type enzyme and gave similar binding constants for eEF-2. Initially, this observation was perplexing because some mutants, namely T442C-, S449C-,
and GDP-concentrations, and the reaction was initiated with 5 nM wild-type squares, 5-AF-labeled-GTP (open squares), 5-AF-labeled-GDP-β-S (open circles), or 5-AF-labeled-GDP-β-S-eEF-2 (open triangles) as described under "Experimental Procedures." The fluorescence excitation was 337 nm, and emission was 460 nm (5 nm band passes) at 25 °C. The raw fluorescence quenching data were converted to fractional saturation values (∆F/∆F_{max}) and are plotted against the 5-AF-labeled eEF-2 concentration. Both GTP-γ-S and GDP-β-S were at 50 μM.

**Table III**

Comparison of the relative ADPRT activity for wild-type PE24H with the various conformational states of eEF-2

| eEF-2 substrate | Relative ADPRT^a |
|-----------------|------------------|
| Native-absence of bound nucleotides | 100 ± 9\(^b\) |
| β-GDP bound | 102 ± 4 |
| γ-GTP bound | 92 ± 10 |

^a The kinetics were determined as described under "Experimental Procedures" and represent the mean ± S.D. of three independent experiments. e-NAD^+ (500 μM) and eEF-2 (14 μM) were held at saturating concentrations, and the reaction was initiated with 5 μM wild-type toxin.

^b The 100% ADPRT activity correlates to a k_{cat} value of 1525 ± 168 min\(^{-1}\) for wild-type PE24H.

and E486C-AEDANS, all exhibited decreased catalytic function (Table II), which, in part, put into question the structural integrity of these proteins. However, when PE24H-AEDANS is completely denatured with urea and eEF-2-AP remains in its native conformation, binding is undetectable (Fig. 3B). Therefore, if PE24H proteins are unfolded or perhaps misfolded, they will be unable to associate, and hence no FRET will occur. This gave credence to this FRET assay as an approach to detecting a specific interaction between toxin and eEF-2 and eliminated the possibility of a fluorophore-fluorophore binding artifact.

Therefore, although some of the labeled mutants had modified ADPRT activity, they still maintained the ability to bind the eEF-2 substrate. Furthermore, it might be expected that the interaction of these two proteins would involve a large number of hydrogen bonds, as well as electrostatic and Van der Waals interactions, and that modification of a single site within the toxin-enzyme would not have a significant impact on the stability of the association complex. The binding constant for the toxin-eEF-2 association was \( K_a \) = 1 μM (Table II), which agrees with earlier findings using analogue-incorporated toxin (27).

A significant finding in the results herein is that the NAD\(^+\) substrate is not essential for the toxin binding with eEF-2, as shown in Fig. 3B. Regardless of the presence or not of the dinucleotide substrate, the two proteins still associate to form a complex of similar stability. This is in agreement with previous work both using the analogue-based FRET assay (27) and through immunoprecipitation experiments (28). Interestingly, NAD\(^+\) had previously been shown to be essential in assays based on immobilized eEF-2 (12, 29). However, the FRET-based assays represent measurements where the proteins are in solution under conditions whereby ADPRT activity can be detected, and thus these data would be expected to more representative of the in vivo behavior for these proteins. This new insight into this enzyme reaction suggests that the ADPRT catalytic mechanism is a random order type, in which binding of either substrate, NAD\(^+\) or eEF-2, occurs followed by the binding of the other substrate. Previously, the reaction was proposed to follow a compulsory order mechanism (11, 12); in light of these new findings, a new enzymatic reaction model is required.

The PE24H ADPRT activity has previously been shown to be sensitive to the ionic strength of the reaction mixture, with the toxin-enzyme capable of normal catalytic function at KCl concentrations less than 100 mM (21, 30), and yet the NAD\(^+\)-PE24H interaction is only modestly dependent on salt concentration (21). It was proposed earlier that the observed salt effect on the activity of PE24H might be a reflection of the influence of ionic strength on the electrostatic interactions involved with eEF-2 (21). However, in the present study, it was shown that the binding event is relatively insensitive to salt levels (Fig. 4A). When the salt concentration was increased to 800 mM, at which level catalytic activity is completely absent (21), relatively little salt dependence for toxin-eEF-2 binding was observed. This is an important finding suggesting that the catalytic residues within the toxin active site are sensitive to electrostatic screening by ions and that the critical catalytic steps likely involve charged substituents within the transition state species for the ADPRT reaction.

This is also the first report that the toxin-eEF-2 interaction is highly dependent on pH (Fig. 4B). This dependence shows a bell-shaped relationship with a pH optimum near 7.8, an acidic pK\(_a\) value near 6.3, and an alkaline pK\(_a\) value near 9.3. Remarkably, this pH binding profile is very similar to the pH dependence observed for the catalytic activity of the toxin (21). Therefore, this pH dependence for activity may be correlated with the eEF-2 binding event. Furthermore, these pK\(_a\) values may give insight into important eEF-2 binding residues within the toxin. The acidic pK\(_a\) value is similar to that usually found for histidine. His-440 has been implicated in eEF-2 binding. However, before the work herein was done, the evidence has been circumstantial. Although His-440 is situated at the base of the active site, its ionization state may be critical for the interaction with the eEF-2 protein substrate. The alkaline pK\(_a\) may be attributed to Tyr-481, which stacks with the nicotinamide portion of the NAD\(^+\) substrate (11), is situated near the site of cleavage within NAD\(^+\), and thus may play an integral part in the transferase event of the catalytic mechanism (8). Moreover, both Tyr-481 and His-440 are highly conserved among diphthamide-targeting ADPRT enzymes (9, 11).

When eEF-2 associates with GTP, a conformational change is induced in the GTP-binding domain of eEF-2. This allows eEF-2 to bind to the pre-translocation ribosome with high affinity. The protein then translocates the growing peptide, which is coupled to the hydrolysis of GTP to GDP. The post-translocation ribosome has a lowered affinity for eEF-2 bound with GDP, thereby releasing this elongation factor from the ribosome (31). Using the intrinsic fluorescence of eEF-2, it has been shown that eEF-2 interacts with GDP, GTP, and their
Interaction of a Bacterial Toxin with Ribosome Translocase

nonhydrolyzable analogues in the absence of ribosomes (23). It was investigated whether the catalytic domain of ETA required a specific conformation of eEF-2 for protein-protein binding to occur. As the results indicate, the toxin did not prefer a specific state of eEF-2 (Fig. 5). Therefore, the toxin is able to bind eEF-2-GTP as it approaches the ribosome before forming the pre-translocation complex, as well as after it has departed the ribosome once GTP has been hydrolyzed. Earlier work using autoradiography had suggested that when NAD \(^-\) and diphtheria toxin (a catalytically similar toxin to ETA) is added to GTP-bound eEF-2, the factor is not ribosylated (32). However, the present work showed that the presence of bound guanyl nucleotides to eEF-2 did not alter the enzyme reaction rate compared with native eEF-2 (Table III). The discrepancy between the two sets of results may rest with the method used for charging the eEF-2 protein with guanyl nucleotides. In the present study, the guanyl nucleotide analogues were simply light-activated to covalently label the GTP-binding site. In the two fragments produced from rat liver eEF-2 digested with endoprotease Glu-C, the N-terminal fragment of eEF-2, which contains the G-domain, maintained its ability to interact with both GTP and GDP, and the C-terminal fragment of eEF-2 was ADP-ribosylated by diphtheria toxin. Because the C-terminal fragment lacks the G-domain, it suggests that this fragment is not governed by the conformational effects of bound guanyl nucleotides and that the critical features within eEF-2 required for toxin recognition and modification are situated within the C terminus of eEF-2. The indifference toward NAD \(^-\) for the enzyme-eEF-2 association process has demonstrated that the current model for the catalytic reaction may, in fact, be a compulsory order mechanism but rather a random order type of mechanism. The pH-dependent toxin-eEF-2 association observed here is similar to the dependence seen for ADFRT catalytic function, thereby suggesting a correlation. Finally, the finding that eEF-2 bound to either GTP or GDP is still recognized as a substrate by the toxin illustrates how highly adaptable this enzyme-toxin is in its mission to ADP-ribosylate eEF-2. Future work is currently under way to elucidate in further detail the location of specific eEF-2-binding sites within the catalytic domain of ETA using this FRET-based and site-specific cysteine-scanning approach.

Acknowledgments—We thank Dr. Victor Marquez for providing the β-TAD compound used in these studies. We also express appreciation to Gerry Prentice for expert technical assistance, including the purification of eEF-2, during the course of this work.

REFERENCES

1. Vasil, M. L. (1986) J. Pediatr. 108, 800–805
2. Van Delden, C., and Iglewski, B. H. (1998) Emerg. Infect. Dis. 4, 551–560
3. Domenighini, M., and Rappuoli, R. (1996) Mol. Microbiol. 21, 667–674
4. Althaus, F. R., and Richter, C. (1987) Mol. Biol. Biochem. Biophys. 37, 1–237
5. Beattie, B. K., Prentice, G. A., and Merrill, A. R. (1996) Biochemistry 35, 15134–15142
6. Sanjal, G., Marquis-Omer, D., Gress, J. O., and Middaugh, C. R. (1993) Biochemistry 32, 3488–3497
7. Beattie, B. K., and Merrill, A. R. (1996) Biochemistry 35, 9042–9051
8. Yates, S. P., and Merrill, A. R. (2001) J. Biol. Chem. 276, 35029–35036
9. Allured, V. S., Collier, R. J., Carroll, S. F., and McKay, D. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1320–1324
10. Wedekind, J. E., Trame, C. B., Dorywalska, M., Koehl, P., Raschke, T. M., McKee, M., FitzGerald, D., Collier, R. J., and McKay, D. B. (2001) J. Biol. Chem. 276, 823–837
11. Li, M., Dyda, F., Benhar, I., Pastan, I., and Davies, D. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9308–9312
12. Kessler, S. P., and Galloway, D. R. (1992) J. Biol. Chem. 261, 19107–19111
13. Li, M., Dyda, F., Benhar, I., Pastan, I., and Davies, D. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6902–6906
14. Oppenheimer, N. J., and Ridley, J. W. (1981) J. Biol. Chem. 256, 8579–8581
15. Giovane, A., Servillo, L., Quagliuolo, L., and Balestrieri, C. (1987) Biochem. J. 244, 337–344
16. Ngymod, O., and Nilsson, L. (1990) Eur. J. Biochem. 191, 1–17
17. Mattheakis, L. C., Shen, W. H., and Collier, R. J. (1992) Mol. Cell. Biol. 12, 4026–4037
18. Iglewski, W. J. (1994) Mol. Cell. Biochem. 138, 131–133
19. Lavergne, J. P., Maranuki, A., Reboud, A. M., and Reboud, J. P. (1990) Biochim. Biophys. Acta 1048, 231–237
20. Perentes, J., Phan, L. D., Gleason, W. B., LaPorte, D. C., Livingston, D. M., and Ridley, J. W. (1992) J. Biol. Chem. 267, 1190–1197
21. Armstrong, S., and Merrill, A. R. (2001) Annu. Rev. Biochem. 70, 226–336
22. Steer, B. A., and Merrill, A. R. (1995) Biochemistry 34, 7225–7233
23. Sontag, B., Reboud, A. M., Divia, G., Di Pietro, A., Guilott, D., and Reboud, J. P. (1993) Biochemistry 32, 1976–1980
24. Wu, P., and Brand, L. (1994) Annu. Rev. Biochem. 63, 1–13
25. Beattie, B. K., and Merrill, A. R. (1990) J. Biol. Chem. 265, 15466–15468
26. Gonzalez, P., Sontag, B., Lavergne, J. P., Jaulit, J. M., and Reboud, J. P. (2000) Biochemistry 39, 13558–13564
27. Mohammadi, F., Prentice, G. A., and Merrill, A. R. (2001) Biochemistry 40, 10273–10283
28. Elzaim, H. S., Chopra, A. K., Peterson, J. W., Goodheart, R., and Heggers, J. P. (1998) Infect. Immun. 66, 2170–2179
29. Prentice, G. A., and Merrill, A. R. (1999) Annu. Rev. Biochem. 72, 216–223
30. Carroll, S. P., and Collier, R. J. (1988) Mol. Microbiol. 2, 293–296
31. Nilsson, L., and Nygård, O. (1988) Eur. J. Biochem. 171, 283–299
32. Nilsson, L., and Nygård, O. (1985) Eur. J. Biochem. 149, 299–304
33. Dumont-Misoepein, A., Lavergne, J. P., and Reboud, J. P. (1995) Biochim. Biophys. Acta 1253, 221–227
34. Lazue, N., and Collier, R. J. (1988) Biochemistry 27, 7629–7632
35. Han, X. Y., and Galloway, D. R. (1995) J. Biol. Chem. 270, 679–684
Insight into the Catalytic Mechanism of *Pseudomonas aeruginosa* Exotoxin A: STUDIES OF TOXIN INTERACTION WITH EUKARYOTIC ELONGATION FACTOR-2

Souzan Armstrong, Susan P. Yates and A. Rod Merrill

*J. Biol. Chem.* 2002, 277:46669-46675.  
doi: 10.1074/jbc.M206916200 originally published online September 20, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M206916200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 12 of which can be accessed free at http://www.jbc.org/content/277/48/46669.full.html#ref-list-1