Elucidation of eukaryotic elongation factor-2 contact sites within the catalytic domain of *Pseudomonas aeruginosa* exotoxin A

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Pseudomonas aeruginosa produces the virulence factor, ETA (exotoxin A), which catalyses an ADP-ribosyltransferase reaction of its target protein, eEF2 (eukaryotic elongation factor-2). Currently, this protein–protein interaction is poorly characterized and this study was aimed at identifying the contact sites between eEF2 and the catalytic domain of ETA (PE24H, an ETA from *P. aeruginosa*, a 24 kDa C-terminal fragment containing a His₆ tag). Single-cysteine residues were introduced into the toxin at 21 defined surface-exposed sites and labelled with the fluorophore, IAEDANS [5-(2-iodoacetylaminomethylamino)-1-naphthalenesulphonic acid]. Fluorescence quenching studies using acrylamide, and fluorescence lifetime and wavelength emission maxima analyses were conducted in the presence and absence of eEF2. Large changes in the microenvironment of the AEDANS [5-(2-aminoethylamino)-1-naphthalenesulphonic acid] probe after eEF2 binding were not observed as dictated by both fluorescence lifetime and wavelength emission maxima values.

This supported the proposed minimal contact model, which suggests that only small, discrete contacts occur between these proteins. As dictated by the bimolecular quenching constant (k_q) for acrylamide, binding of eEF2 with toxin caused the greatest change in acrylamide accessibility (> 50 %) when the fluorescence label was near the active site or was located within a known catalytic loop. All mutant proteins showed a decrease in accessibility to acrylamide once eEF2 bound, although the relative change varied for each labelled protein. From these data, a low-resolution model of the toxin–eEF2 complex was constructed based on the minimal contact model with the intention of enhancing our knowledge on the mode of inactivation of the ribosome translocase by the *Pseudomonas* toxin.

Key words: acrylamide quenching, bacterial toxins, eukaryotic elongation factor 2, exotoxin A, protein–protein interaction.

INTRODUCTION

The ubiquitous Gram-negative bacterium, *Pseudomonas aeruginosa*, is an opportunistic human pathogen that secretes a number of potent virulence factors, including ETA (exotoxin A). The production of these virulence factors allows the bacterium to adapt to many distinct environments such as soil, water, sewage and hospitals. People at a high risk for *P. aeruginosa* infections include those suffering from AIDS, cystic fibrosis, cancer, severe trauma or burns. ETA is a highly virulent protein that largely contributes to the local tissue damage, bacterial invasion and immunosuppression that occurs within the eukaryotic host [1].

ETA is a member of the enzyme family termed mono-ADP-ribosyltransferases and is specifically a NAD⁺-diphthamide ADPRT (ADP-ribosyltransferase, EC 2.4.2.36) [2,3]. ETA is a 66 kDa extracellular protein [4] that enters eukaryotic cells via receptor-mediated endocytosis [5] on route to the cytoplasm where it catalyses the ADP-ribosylation of eEF2 (eukaryotic elongation factor 2) [6]. Inactivation of eEF2 results in cessation of protein synthesis with eventual cellular death [4].

The docking site for the NAD⁺ substrate within the catalytic domain of ETA is well characterized; however, the eEF2 substrate binding and contact sites remain an enigma. The known catalytic residues within ETA include Glu-553, His-440, Tyr-481 and Tyr-470 [7–10]. In the structure of the catalytic domain of ETA, 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 [8]. The nicotinamide ring of NAD⁺ stacks with Tyr-481 and Tyr-470 and

these residues are also involved in van der Waals interactions with the thiazole-ribose moiety of the non-hydrolysable NAD⁺ analogue, β -TAD (β -methylene-thiazole-4-carboxamide adenine dinucleotide). Glu-553 forms a hydrogen bond with the oxygen of the thiazole-ribose of β -TAD [8,11] and maintains NAD⁺ in the correct conformation to allow exposure of the scissile N-glycosidic bond, and the negatively charged carboxylate of Glu-553 may stabilize a positively charged reaction intermediate [8].

eEF2, a member of the GTPase superfamily, is a single polypeptide chain with a molecular mass of approx. 96 kDa [12]. eEF2 is a translation factor responsible for the elongation phase of eukaryotic protein synthesis, catalysing 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 movement of the mRNA to allow the next codon to be placed in the A site [13]. eEF2 contains a post-translationally modified histidine residue, 2-[3-carboxyamido-3-(trimethylammonio)-propyl]histidine, referred to as diphthamide [14,15], which is the specific ribosylation target of ETA. This residue is located at position 699 in the yeast eEF2 sequence near the C-terminus of the protein [16]. Diphthamide has only been found in eEF2 and is completely conserved throughout all eukaryotic and archaeobacterial evolution [15,17]. Although the diphthamide is the site of toxin attack, it does not appear to be essential for eEF2 function but may be a site for regulation of translocase activity.

Some of the physiological conditions necessary for complexation of eEF2 to the catalytic domain of ETA (PE24H, an ETA from *P. aeruginosa*, a 24 kDa C-terminal fragment containing

Abbreviations used: ADPRT, ADP-ribosyltransferase; AEDANS, 5-(2-aminoethylamino)-1-naphthalenesulphonic acid; β -TAD, β -methylene-thiazole-4-carboxamide adenine dinucleotide; DTT, dithiothreitol; eEF2, eukaryotic elongation factor 2; ETA, exotoxin A; IAEDANS, 5-(2-iodoacetylaminomethylamino)-1-naphthalenesulphonic acid; MIANS, 2-(4'-maleimidyl)anilino)naphthalene-6-sulphonic acid.

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a His₆ tag) have previously been characterized. PE24H encompasses the catalytic domain of ETA. This association is pH-dependent, yet insensitive to ionic strength and to the presence of the other substrate, NAD⁺ [18]. Unfortunately, the location of the specific eEF2 contact sites on PE24H remains elusive. The present study was aimed at locating potential contact sites between these proteins by creating a series of PE24H mutant proteins, each uniquely labelled at a specific site with the AEDANS [5-(2-aminoethylamino)-1-naphthalenesulphonic acid] fluorescent probe. Each of these fluorescently tagged proteins was studied using fluorescence lifetime and wavelength emission maxima analyses along with fluorescence quenching studies using acrylamide in the presence and absence of bound eEF2 to disclose any region(s) on PE24H that are important for this protein–protein interaction. Based on the fluorescence results, a model for the toxin–eEF2 complex is proposed that provides the basis for further investigation and understanding of this important protein–protein interaction.

EXPERIMENTAL

Cysteine-scanning mutagenesis

In addition to the previously reported single-cysteine mutant proteins [S408C (Ser⁴⁰⁸ → Cys), S410C, T442C, S449C, S459C, E486C, S507C, S515C and S585C] [18], 12 new single-cysteine mutant proteins (Q415C, Q428C, A476C, R490C, A519C, G525C, G549C, T554C, T564C, N577C, Q592C and Q603C) were created using standard Quik ChangeTM mutagenesis (Stratagene, La Jolla, CA, U.S.A.). The DNA template was the plasmid pPH1 containing the gene for wild-type PE24H and the desired mutation was determined by dideoxy sequencing with an ABI Prism Model 377 DNA sequencer using dye termination and cycle sequencing and was analysed on a 4.5 % (w/v) acrylamide gel.

Overexpression and purification of PE24H

The catalytic fragment of ETA with a C-terminal His₆ tag (PE24H) was overexpressed and purified as described in [19], except that all cysteine-containing mutant proteins required the chelate-agarose affinity column to be charged with 100 mM ZnSO₄.

Fluorescence labelling of PE24H

The PE24H protein was labelled with the fluorophore IAEDANS [5-(2-iodoacetylaminomethylamino)-1-naphthalenesulphonic acid] as detailed earlier [20] with some modifications. The labelling efficiency, based on the appropriate absorbances and molar absorption coefficients (ϵ) of the bound fluorophore and the measured protein was determined.

The S410C, Q415C and G525C mutant toxins were also labelled with the fluorescence reagent, MIANS [2-(4'-maleimidylanilino)naphthalene-6-sulphonic acid]. Briefly, 0.5 mg of purified PE24H protein was incubated in 200 mM Tris/HCl (pH 8.1) with an excess of DTT (dithiothreitol; molar ratio of 5:1, mol DTT/mol PE24H) at room temperature (25 °C) for 30 min. A concentrated solution of MIANS (in DMSO) was added to the reaction mixture [5 % (v/v) DMSO, final concentration] to give a molar ratio of 10:1 (MIANS/PE24H) and the reaction was gently mixed on a nutator for 30 min at room temperature. The reaction was quenched by the addition of an excess of DTT (100 mM, final concentration). The quenched sample was loaded on to a prepacked BioRad Econo-Pac 10DG column (Bio-Rad Laboratories, Mississauga, ON, Canada) and the protein adduct was

eluted with 50 mM NaCl, 20 mM Tris/HCl (pH 7.9) buffer. The protein-adduct peak was well separated from the free-dye peak. The labelled PE24H protein was further characterized by UV spectroscopy using $\epsilon_{M(280)nm} = 27\,310\text{ M}^{-1} \cdot \text{cm}^{-1}$ for PE24H and $\epsilon_{M(322)nm} = 20\,877.2\text{ M}^{-1} \cdot \text{cm}^{-1}$ for MIANS [21]. The labelling efficiency, based on the appropriate absorbances and ϵ values of the bound fluorophore and the measured protein, was nearly 100 %.

Purification of eEF2

eEF2 was purified from wheat germ as described previously in [19] with some modifications.

Steady-state fluorescence

All steady-state fluorescence measurements were obtained using a PTI Alphascan spectrophotometer interfaced with a computer using FelixTM version 1.21 software (Photon Technology International, South Brunswick, NJ, U.S.A.) and equipped with a water-jacketed sample chamber set to 25 °C.

Fluorescence-based ADPRT assay

The NAD⁺-dependent ADPRT activity for the new cysteine PE24H proteins and their corresponding protein adducts was determined as described by Armstrong et al. [19].

Substrate-binding assays

Both the eEF2-binding [20] and the NAD⁺-binding assays [4] have been described previously and were used to characterize the binding activities of the single-cysteine mutant PE24H proteins.

Tryptophan fluorescence emission spectra

Fluorescence emission spectra of the 21 single-cysteine PE24H proteins (1.0 μM in a volume of 600 μl) in 50 mM NaCl, 20 mM Tris/HCl (pH 7.9) buffer were acquired by excitation at 295 nm and the emission was monitored from 310 to 450 nm with all slits held at 4 nm. The fluorescence λ emission maxima ($\lambda_{em,max}$) were determined for each mutant toxin from the buffer-corrected spectra. These data were used to assess the structural integrity of the folded mutant proteins as compared with the wild-type protein.

Determination of $\lambda_{em,max}$ for AEDANS-labelled toxins

The fluorescence emission spectra of the 21 AEDANS-labelled toxins (1.0 μM) in 50 mM NaCl, 20 mM Tris/HCl (pH 7.9) buffer were excited at 337 nm and the emission was monitored from 400 to 625 nm with all slits held at 4 nm. eEF2 (6.0 μM) was subsequently added and allowed to incubate with the toxin for 5 min before determining the emission spectra in the presence of eEF2. The $\lambda_{em,max}$ values were determined for each AEDANS-labelled toxin in the absence and presence of eEF2 from the buffer-corrected spectra.

Acrylamide quenching

Acrylamide stocks (5 M) were prepared in 50 mM NaCl, 20 mM Tris/HCl (pH 7.9) buffer. The concentration of acrylamide was determined using $\epsilon = 0.25\text{ M}^{-1} \cdot \text{cm}^{-1}$ at 295 nm [22] using a CARY 300 Varian absorbance spectrophotometer (Varian Canada, Mississauga ON, Canada).

The AEDANS toxin (1.0 μM in 50 mM NaCl/20 mM Tris/HCl, pH 7.9) was titrated with small aliquots of acrylamide ranging from 0 to 0.3 M in ultramicrocuvettes with a starting volume

of 70 μ l. Fluorescence measurements were conducted with 337 nm excitation light and the emission was monitored at 492 nm. Excitation and emission slit widths ranged from 4 to 6 nm. The fluorescence signal was collected for 30 s and the data were analysed by integrating the area under the 30 s time trace. All experiments were performed at 25 °C. Experiments were repeated in the presence of 6.0 μ M eEF2 after a 5 min incubation. Quenching data were plotted as the ratio of fluorescence intensity in the absence of quencher (F_0) to the intensity in the presence of quencher (F_i) against quencher concentration. The slope was equated to dynamic parameters according to the modified Stern–Volmer equation $F_0/F_i = 1 + K_{SV}[\text{acry}]$, where K_{SV} is equal to $k_q\tau_0$. K_{SV} , k_q and τ_0 are respectively, the Stern–Volmer quenching constant, bimolecular quenching constant and fluorescence lifetime in the absence of quencher, and $[\text{acry}]$ represents the concentration of acrylamide. For mutant PE24H proteins with reduced affinity for eEF2, the distribution of free and bound toxin was accounted for in the acrylamide experiments in the presence of eEF2. This is described by the expression [23]:

$$\frac{F_0}{F_i} = (\tau_0 K_D + \tau_0' [\text{eEF2}]) \left\{ \frac{\tau_0 K_D}{(1 + k_q \tau_0 [\text{acry}]) + \frac{\tau_0' [\text{eEF2}]}{1 + k_q' \tau_0' [\text{acry}]}} \right\}^{-1}$$

where K_D is the eEF2-binding constant, τ_0' the lifetime of eEF2-bound toxin and k_q' the bimolecular quenching constant of eEF2-bound toxin.

Temperature-dependent studies were performed to deduce whether the quenching of PE24H-AEDANS by acrylamide was either dynamic (collisional) or static. Acrylamide quenching was performed at 10, 20, 25 and 30 °C. A collisional quenching mechanism is expected to show increases in the K_{SV} value with temperature, whereas the static mechanism will show decreases in K_{SV} as the temperature increases.

To evaluate the potential viscosity effect that may result from the addition of eEF2 to the protein solution, the level of eEF2 was increased to twice the saturating concentration (12 μ M) and the acrylamide quenching experiments were repeated.

Acrylamide quenching for S410C-, Q415C- and G525C-MIANS was as stated for AEDANS-PE24H proteins, except that the fluorescence measurements were performed with 322 nm excitation light and the emission monitored at 420 nm. Excitation and emission bandpasses were held at 4 and 6 nm respectively.

Fluorescence lifetime measurements

Time-resolved fluorescence measurements were performed using a PTI LaserStrobe model GL3300 (Photon Technology International). The excitation source was a pulsed nitrogen laser operating at 10 Hz and a dye laser attached to a frequency doubler. The fluorescence decay, following excitation at 337 nm, was acquired with the emission set to 480 nm. The emission slit-width was set to 4 nm and the temperature was 25 °C. Data were collected at 161 ps/channel in 500 channels in an 80.5 ns time window as the average of 24 signals/channel. The instrument response function was determined from a scattering solution of colloidal silica (LudoxTM). The fluorescence lifetimes were determined for each AEDANS-labelled toxin in the absence and presence of eEF2.

The quality of the exponential decay global fits was assessed by inspection of the plots of weighted residuals and by the statistical parameters χ^2 and SVR (serial variance ratio). The reduced χ^2 for the global fit ($\chi^2 = 1$ for an ideal fit) ranged from 0.9 to 1.2 for all samples.

RESULTS

Single-cysteine PE24H mutant proteins and the integrity of their folded structures

PE24H contains no endogenous cysteine residues; therefore, single-cysteine residues were introduced at defined surface-exposed locations within the toxin. Nine single-cysteine mutant proteins have been described previously [18]. In addition, 12 new single-cysteine toxins were created to ensure that the entire surface of the toxin protein was probed for the interaction with eEF2. Since electrostatic interactions are often important for protein–protein interactions [24], only uncharged residues were changed into cysteine. These included polar uncharged residues (serine, threonine, glutamine and asparagine) and small uncharged residues (alanine and glycine). The exceptions are E486C and R490C, both members of a previously identified catalytic loop situated near the active site to assess the involvement of this element in eEF2 binding [20]. The locations of the 21 sites chosen are shown in Figure 1. Each protein was subsequently labelled with IAEDANS as described previously [20].

The folded integrity of each of the mutant PE24H proteins was assessed by tryptophan fluorescence spectroscopy as reported by the three tryptophan residues that are situated within or near the active site of the enzyme. The $\lambda_{\text{em,max}}$ value for the single-cysteine PE24H proteins ranged from 330 to 334 nm, which is similar to the wild-type enzyme, and which exhibits a $\lambda_{\text{em,max}}$ value of 331 nm [25]. It was previously shown that a strong correlation exists between the $\lambda_{\text{em,max}}$ values and the secondary structure (CD spectra) for the PE24H protein [26]. Furthermore, it is conceivable that the attachment of the AEDANS fluorophore could result in the local perturbation of the protein structure that may skew some of the interpretation of the PE24H contact sites with eEF2. This is an inherent weakness in the use of extrinsic fluorescence probes to study the protein structure. However, large changes in the structure and behaviour (enzyme activity as described below) were not observed for most of the AEDANS-labelled single-cysteine mutant proteins (except for those residues known to be involved in or to consist of a part of the enzyme structure participating in the catalytic mechanism).

Each PE24H mutant protein and its AEDANS adduct was evaluated by its ADPRT activity relative to the wild-type protein. Generally, the proteins did not show a significant loss of function (1–6-fold loss of activity relative to wild-type; Table 1). However, the mutant proteins T442C-, S449C-, E486C-, T554C- and A519C-AEDANS were the exceptions, as they did show an alteration in enzyme function (12–100-fold loss; Table 1). As reported previously, 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-bonded with NAD⁺ [8,11], and Glu-486 is located within a catalytic loop region [20]. The integrity of these mutants was assessed in a previous study [18], and shown to retain the ability to bind eEF2 despite a reduction in enzyme activity. T554C is situated on the same β -strand as Glu-553, an important catalytic residue. The side chain of Thr-554 projects away from the active site, yet cysteine replacement of this residue may alter the required alignment of the catalytic Glu-553 to contact NAD⁺. The substitution of this glutamate residue with aspartate shows a significant reduction in enzymic activity [27]. A519C is in a loop region near the entrance of the active site. After the attachment of the fluorescent probe, A519C retained only 5% of wild-type activity, thereby suggesting that the probe may interfere with substrate accessibility to the enzyme active site.

Interestingly, A519C-AEDANS retained the ability to bind eEF2 with normal affinity (the dissociation constant $K_D = 0.9 \pm 0.1$ μ M). However, T554C-AEDANS was unique in that it showed

Figure 1 Structure of the catalytic domain of ETA complexed with an NAD⁺ analogue

The bound NAD⁺ analogue (β -TAD) is shown in stick form and the protein backbone is shown in ribbon format. The 21 sites that were substituted with cysteine residues by site-directed mutagenesis are shown as space-filled structures. These residues were subsequently labelled with the fluorescence reagent IAEDANS to create 21 singly labelled toxins. The model was prepared with the desktop PC modelling package, DS Viewer Pro version 5.0 (Accelrys, San Diego, CA, U.S.A.). The full co-ordinates of the catalytic domain of PE24H- β -TAD complex (PDB entry, 1AER) [8] were obtained from the RCSB Protein Data Bank (<http://www.rcsb.org/pdb/>).

a weak binding affinity for the eEF2 substrate ($K_D = 5.7 \pm 1.3 \mu\text{M}$). For this mutant protein, affinity for the NAD⁺ substrate was also reduced; K_D values for T554C and T554C-AEDANS were 209 ± 22 and $578 \pm 53 \mu\text{M}$ respectively, whereas the wild-type K_D is $55 \mu\text{M}$ [20]. This questioned the integrity of the active site within this mutant enzyme. However, as alluded to above, the $\lambda_{\text{em,max}}$ value for T554C was 333 nm, which is similar to the wild-type enzyme [25]. These data suggest that T554C is not largely misfolded, but may suffer from a local perturbation within the active site, whereby the position of the catalytic residue Glu-553 may be altered and may further change the disposition of the proposed eEF2 substrate-binding loop within the active site of the toxin [28].

Steady-state fluorescence

The polarity of the environment around the AEDANS probe was assessed by steady-state fluorescence measurements since spectral properties often provide information regarding the local environment of this probe. The $\lambda_{\text{em,max}}$ for the emission spectrum of AEDANS has previously been reported to range from 520 nm in water to 448 nm in dimethylformamide [29]. Hence, shorter $\lambda_{\text{em,max}}$ wavelengths correlate to the AEDANS probe being situated in a relatively non-polar environment, whereas longer wavelengths indicate that the probe is situated in a more polar environment.

Table 2 shows the $\lambda_{\text{em,max}}$ values of the corrected fluorescence emission spectra for the 21 AEDANS-PE24H toxins both in the absence and in the presence of eEF2. The $\lambda_{\text{em,max}}$ values ranged from 471 nm for T442C-AEDANS to 483 nm for R490C-AEDANS and these correspond to the relative polarity of their environments and location within the PE24H structure. For instance, T442C-AEDANS is located at the base of the active-site cleft and R490C-AEDANS is relatively exposed on the tip of a loop region. It was expected that, after the addition and subsequent binding of eEF2 to the toxin, spectral shifts would result owing to a change in the local environment of the AEDANS probe. This is a consequence of the large eEF2 protein docking on to the toxin surface at a site near the fluorescent probe. However, as Table 2 indicates, no large shifts were observed, indicating that the environment of the AEDANS probe did not significantly change after the addition of eEF2. The largest observed changes include a 3 nm red-shift for both S449C- and S515C-AEDANS and a 1 nm blue-shift for A519C-AEDANS. Thus the binding of eEF2 slightly increased the polarity of the AEDANS probe at residues 449 and 515 (although the changes are not large, they are significant). Two possibilities exist for this phenomenon. First, the protein-protein interaction may induce a protein conformational change near residues 449 and/or 515, which may involve an increased exposure to the aqueous solvent. Alternatively, eEF2 binds at or near these two residues and the increase in polarity

Table 1 Comparison of the ADPRT activity for the cysteine-replacement mutants and their AEDANS and MANS derivatives

Values are given relative to the wild-type protein. The ADPRT value for the wild-type was $878 \pm 27 \text{ min}^{-1}$ and was determined as described in the Experimental section. Values are the means $\pm$ S.D. for three independent experiments.

PE24H	Relative ADPRT activity		
	Native protein	AEDANS adduct	MANS adduct
- Wild-type	1.00		
1 S408C	$0.93 \pm 0.17^*$	$0.64 \pm 0.07^*$	
2 S410C	$0.27 \pm 0.05^*$	$0.54 \pm 0.10^*$	0.14 ± 0.01
3 Q415C	0.48 ± 0.04	0.33 ± 0.01	0.19 ± 0.02
4 Q428C	0.54 ± 0.03	0.36 ± 0.02	
5 T442C	$0.20 \pm 0.05^*$	$0.01 \pm 0.002^*$	
6 S449C	$0.35 \pm 0.07^*$	$0.06 \pm 0.01^*$	
7 S459C	$0.46 \pm 0.17^*$	$0.80 \pm 0.11^*$	
8 A476C	0.51 ± 0.04	0.37 ± 0.02	
9 E486C	$1.38 \pm 0.21^*$	$0.08 \pm 0.01^*$	
10 R490C	0.18 ± 0.02	0.39 ± 0.03	
11 S507C	$1.86 \pm 0.12^*$	$0.40 \pm 0.05^*$	
12 S515C	$0.45 \pm 0.12^*$	$0.59 \pm 0.12^*$	
13 A519C	0.36 ± 0.01	0.05 ± 0.002	
14 G525C	1.03 ± 0.07	0.71 ± 0.10	0.44 ± 0.05
15 G549C	0.16 ± 0.01	0.24 ± 0.03	
16 T554C	0.02 ± 0.002	0.02 ± 0.001	
17 T564C	1.09 ± 0.10	0.19 ± 0.01	
18 N577C	0.31 ± 0.01	0.43 ± 0.06	
19 S585C	$1.08 \pm 0.23^*$	$0.28 \pm 0.09^*$	
20 Q592C	0.80 ± 0.05	0.81 ± 0.11	
21 Q603C	0.67 ± 0.03	0.73 ± 0.06	

*Data taken from Armstrong et al. [18].

Table 2 Fluorescence lifetime (τ_0) and fluorescence emission maxima ($\lambda_{\text{em,max}}$) values of the various PE24H-AEDANS proteins in the absence and presence of eEF2

The fluorescence parameters were determined as described in the Experimental section. All τ_0 values were single exponential decay curves. Errors for the $\lambda_{\text{em,max}}$ values were all $< 0.5 \%$.

Protein adduct	τ_0 (ns)		$\lambda_{\text{em,max}}$ (nm)	
	- eEF2	+ eEF2	- eEF2	+ eEF2
1 S408C	13.8 ± 0.4	13.6 ± 0.5	481	481
2 S410C	14.4 ± 0.1	14.5 ± 0.2	479	479
3 Q415C	14.3 ± 0.3	13.9 ± 0.3	478	479
4 Q428C	15.9 ± 0.5	14.7 ± 0.1	478	479
5 T442C	16.9 ± 0.5	16.8 ± 0.4	471	472
6 S449C	15.2 ± 0.1	15.7 ± 0.9	473	477
7 S459C	14.4 ± 0.5	13.9 ± 0.5	479	480
8 A476C	15.9 ± 0.2	15.6 ± 0.8	478	478
9 E486C	14.9 ± 0.1	14.6 ± 0.6	478	479
10 R490C	12.0 ± 0.5	11.7 ± 0.3	483	483
11 S507C	13.2 ± 0.2	13.7 ± 0.4	482	481
12 S515C	16.0 ± 0.2	15.4 ± 0.3	473	476
13 A519C	15.7 ± 0.1	16.9 ± 0.1	479	478
14 G525C	13.4 ± 0.1	13.4 ± 0.3	481	481
15 G549C	15.5 ± 0.4	15.8 ± 0.2	480	479
16 T554C	15.3 ± 0.1	15.9 ± 0.8	478	477
17 T564C	16.7 ± 0.3	16.5 ± 0.2	474	475
18 N577C	12.7 ± 0.1	12.4 ± 0.4	482	482
19 S585C	14.0 ± 0.2	14.2 ± 0.6	481	481
20 Q592C	15.2 ± 0.4	14.9 ± 0.6	478	479
21 Q603C	14.2 ± 0.1	14.0 ± 0.7	481	482

may be due to polar and/or charged residues directly contacting the fluorescent probe. Conversely, A519C-AEDANS is in an exposed loop and the 1 nm blue-shift is probably a direct result of contact

with eEF2 causing a slight decrease in the polarity around the AEDANS probe at residue 519.

Time-resolved fluorescence

The fluorescence lifetime for the 21 mutants was also determined (Table 2). Similarly, as for the $\lambda_{\text{em,max}}$ values, the τ_0 values can give information regarding the environment of the polarity for the fluorescence probe, and may also provide valuable data on the conformational and segmental dynamics of the protein [30]. Interestingly, all mutant proteins displayed single-exponential fluorescence decay profiles, indicating the general homogeneity of the AEDANS probe environment. The fluorescence decay parameters ranged from 12 to 17 ns for R490C- and T442C-AEDANS respectively (Table 2). As for the $\lambda_{\text{em,max}}$ data, large changes in the τ_0 values were not observed after toxin binding with eEF2. The τ_0 value of Q428C-AEDANS showed a 1.2 ns decrease and A519C-AEDANS showed a 1.2 ns increase with eEF2 binding. Residue 428 is situated in a helix that is distant from the active site, and a previous work [26] has demonstrated that this helix is not important for eEF2 binding and its role is to maintain the tertiary structure and fold of the toxin. Therefore the change in τ_0 for Q428C-AEDANS is probably owing to an induced conformational change at that site rather than direct contact with eEF2. In contrast, both the $\lambda_{\text{em,max}}$ and the τ_0 values indicate that a decrease in polarity accounts for the structural changes occurring within the AEDANS environment at position 519.

Acrylamide quenching of PE24H-AEDANS proteins

Since the $\lambda_{\text{em,max}}$ and τ_0 parameters did not show large changes, acrylamide quenching was employed to provide additional data on the nature and location of the contact regions between the two proteins. Acrylamide is a non-ionic quencher, which can give information regarding the solvent accessibility of the AEDANS fluorescence probe. It has been shown previously that acrylamide has the ability to quench the fluorescence of an extrinsic probe like AEDANS [31,32]. Each of the PE24H-AEDANS proteins was titrated with acrylamide, in the absence and presence of bound eEF2, and Stern–Volmer plots were constructed. The titration curves were linear between 0 and 0.3 M acrylamide and were fitted by least-squares linear regression analysis. From the Stern–Volmer plots, the slope is the Stern–Volmer constant K_{SV} ; however, since the decay time parameters for the AEDANS-PE24H proteins varied, the K_{SV} values of the mutants cannot be compared directly. Thus the bimolecular quenching constant (k_q) was calculated by dividing the K_{SV} parameter by the fluorescence lifetime (K_{SV}/τ_0), thereby allowing comparison of the relative solvent accessibility of the AEDANS probe at various sites on the surface of the toxin. The k_q values ranged from 0.50 (T564C-AEDANS) to $1.42 \text{ M}^{-1} \cdot \text{ns}^{-1}$ (Q592C-AEDANS) in the absence of eEF2 and from 0.16 (T554C-AEDANS) to $0.79 \text{ M}^{-1} \cdot \text{ns}^{-1}$ (Q592C-AEDANS) in the presence of eEF2 (Table 3). All mutant proteins showed a decrease in accessibility to acrylamide on binding eEF2, although the relative change varied for each labelled protein. When determining k_q in the presence of eEF2, the distribution of free and eEF2-bound toxin was accounted for based on the measured K_D value for this interaction (approx. $1 \mu\text{M}$). The calculation of k_q when adjusted for the individual K_D values gave similar values to the unadjusted k_q value (results not shown), indicating that the toxin is predominantly in the bound state for most of the mutant protein–eEF2 complexes. The exception is T554C-AEDANS, which is the only mutant toxin that exhibited significantly reduced binding affinity for eEF2 (a 6-fold decrease). In this case, the k_q decreased from 0.32 to $0.16 \text{ M}^{-1} \cdot \text{ns}^{-1}$.

Table 3 The bimolecular quenching constant (k_q) values for the PE24H-AEDANS proteins in the absence and the presence of eEF2 as determined by acrylamide quenching experiments

The fluorescence parameters were determined as described in the Experimental section. The calculation of k_q in the presence of eEF2 for T554C-AEDANS accounted for the distribution of free and bound toxin, as this mutant protein possesses decreased binding affinity for eEF2 (see text for further discussion).

Protein adduct	$k_q (\times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1})$	
	– eEF2	+ eEF2
1 S408C	1.03 ± 0.02	0.37 ± 0.01
2 S410C	1.29 ± 0.01	0.36 ± 0.01
3 Q415C	1.28 ± 0.04	0.73 ± 0.01
4 Q428C	0.86 ± 0.03	0.54 ± 0.01
5 T442C	0.56 ± 0.02	0.20 ± 0.01
6 S449C	0.60 ± 0.02	0.27 ± 0.01
7 S459C	0.94 ± 0.03	0.42 ± 0.02
8 A476C	0.89 ± 0.03	0.58 ± 0.01
9 E486C	1.29 ± 0.04	0.48 ± 0.02
10 R490C	1.27 ± 0.04	0.70 ± 0.02
11 S507C	0.80 ± 0.02	0.33 ± 0.01
12 S515C	0.55 ± 0.03	0.41 ± 0.01
13 A519C	1.23 ± 0.04	0.61 ± 0.02
14 G525C	0.96 ± 0.01	0.75 ± 0.02
15 G549C	0.65 ± 0.03	0.35 ± 0.01
16 T554C	0.88 ± 0.04	0.16 ± 0.01
17 T564C	0.50 ± 0.02	0.27 ± 0.01
18 N577C	1.10 ± 0.04	0.74 ± 0.01
19 S585C	0.90 ± 0.02	0.59 ± 0.01
20 Q592C	1.42 ± 0.02	0.79 ± 0.01
21 Q603C	0.68 ± 0.02	0.39 ± 0.01

Figure 2 Comparison of k_q values determined from acrylamide quenching of the various PE24H-AEDANS proteins in the absence and presence of eEF2

Samples were prepared in 50 mM NaCl, 20 mM Tris/HCl (pH 7.9) buffer and were excited at 337 nm. k_q was determined from the Stern–Volmer equation $(F_0/F_1) = K_{SV} + 1$, where $K_{SV} = k_q \tau_0$. The exception is T554C-AEDANS (*), which has a decreased binding affinity for eEF2; thus the distribution of free and bound toxin must be accounted for to determine accurately k_q values in the presence of eEF2 (see the Experimental section for details of this calculation).

The ratio of k_q in the absence and presence of eEF2 is shown in Figure 2. The mutant proteins that exhibited a > 50 % reduction in k_q after the addition of eEF2 include (from highest to lowest) T554C-, S410C-, S408C-, T442C-, E486C, S507C-, S459C-,

Figure 3 Stern–Volmer plot of PE24H-AEDANS in the presence of 6 and 12 μM eEF2 to determine whether the addition of eEF2 introduced a viscosity effect

The fluorescence quenching of PE24H-AEDANS in the presence of 6 μM eEF2 (Δ) or 12 μM eEF2 ($\circ$) by acrylamide with excitation at 337 nm and emission at 492 nm (4 nm bandpasses) at 25 °C and solution conditions as described in Figure 2.

S449C- and A519C-AEDANS. Generally, accessibility of the AEDANS probe to acrylamide after eEF2 binding decreased if the site-specific probe was situated within or near the opening of the active site.

Since all mutant proteins showed a decrease in k_q after eEF2 binding, it was anticipated that the addition of eEF2 may introduce a non-specific viscosity effect. The eEF2 protein is a large biomolecule (approx. 96 kDa) and simply the addition of eEF2 (as a polyelectrolyte) could alter the viscosity of the medium, which in turn would lower the diffusion of acrylamide through the medium to subsequently quench the fluorescence probe. Temperature-dependent studies showed that the quenching follows a collisional quenching mechanism, as the K_{SV} value increased with temperature (results not shown). Therefore acrylamide quenching was performed at 6 and 12 μM to determine whether the higher concentration of eEF2 caused the K_{SV} value to decrease even further due to a viscosity effect. Figure 3 shows the Stern–Volmer plot at these two different eEF2 concentrations. The K_{SV} value was not affected by the increase in eEF2 concentration indicating that the change in viscosity is probably minimal and does not affect the ability of acrylamide to collide with and quench the AEDANS probe.

In addition, to ensure that the results for the acrylamide quenching of the site-specific probes was not limited to the AEDANS moiety, acrylamide quenching was performed on MIANS-labelled PE24H proteins. MIANS was chosen since it has a similar molecular mass as IAEDANS (molecular masses of IAEDANS and MIANS are 0.434 and 0.416 kDa respectively) and a similar but not identical structure. Acrylamide quenching of MIANS-labelled lactose permease has previously been reported in [33]. Three mutant proteins were chosen based on the AEDANS quenching experiments and represented those that showed a range of changes in k_q after eEF2 binding. According to the data for the AEDANS-acrylamide study, S410C, Q415C and G525C showed a reduction in their K_{SV} values, by 72, 43 and 22 % respectively. Importantly, as seen in Figure 4, the same trends in the K_{SV}

Figure 4 Comparison of the quenching of the PE24H fluorescence adducts AEDANS and MIANS by acrylamide

The S410C, Q415C and G525C adducts are representative of proteins that showed large, moderate and small changes respectively in K_{sv} values after the addition of eEF2 (as determined from the original AEDANS-PE24H acrylamide experiments). The MIANS probe was used to determine whether or not the quenching of AEDANS by acrylamide was fluorophore-specific. Black bars, PE24H-AEDANS proteins; grey bars, PE24H-MIANS.

values were observed for the MIANS-labelled proteins as for the AEDANS-labelled proteins.

DISCUSSION

Two extreme models may be considered for the interaction of toxin with eEF2, namely minimum- versus maximum-contact models. In the minimum-contact model, the number of contact locations between these proteins is limited and these sites may be located probably in and around the active site of the toxin. The alternative is the maximum-contact model where eEF2, a much larger protein when compared with PE24H (96 versus 24 kDa), may partially or completely engulf the toxic protein; hence sites of contact would not be limited to the active-site region but may include quite distant sites. Initially, this study began in the absence of a three-dimensional structure for eEF2; therefore, both models were assumed to be possible.

The spectral properties, $\lambda_{em,max}$ and τ_0 values, of the 21 different PE24H-AEDANS proteins gave insight into the microenvironment of the probe in the various locations, and these parameters correlated well with the relative location of the probes within the X-ray structure of the toxin (Table 2 and Figure 1). However, after the addition of eEF2, no significant alterations in these spectral parameters were observed (Table 2). This was surprising, since the probe, AEDANS, is very sensitive to its local environment. These results completely supported the minimal-contact model, suggesting that only small, discrete interactions were occurring between eEF2 and the toxin, thereby not altering the microenvironment of the probes significantly. Both fluorescence resonance energy transfer based [18,34] and immuno-precipitation approaches [35] have shown that these proteins do specifically associate in solution. However, these results did not provide sufficient information regarding specific regions on the toxin that may be involved in the contact with eEF2.

Therefore, to survey further the site(s) of association, the ability of acrylamide to quench the fluorescence of the AEDANS probe was evaluated. If the fluorescent probe becomes more accessible to acrylamide, a greater degree of quenching will be observed. The AEDANS probe would be expected to be less accessible to acrylamide (protected) if eEF2 binds at that region on the toxin. Thus a decrease in solvent accessibility (as witnessed by a decrease in accessibility to the quencher, acrylamide) will be reflected by a reduction in the k_q value after the addition of eEF2. The largest decreases in the k_q values (a change of > 50%) after the addition of eEF2 (Figure 2) were found for T554C-, S410C-, S408C-, T442C-, E486C-, S507C-, S459C-, S449C- and A519C-AEDANS. All of these residues, except S507C-AEDANS, are close to the active site or near the catalytic loop region (residues 483–490) [20] (Figure 1) and hence add further credence to the minimal-contact model proposed. Both S410C- and S408C-AEDANS are located on a short β -strand near the vicinity of the catalytic loop region and may either contact eEF2 directly or the movement of this loop may cause the accessibility of Ser-408 and Ser-410 to decrease on eEF2 binding. E486C-AEDANS, a member of this loop, was also affected by eEF2 binding. S459C- and S449C-AEDANS both surround the opening to the active site. T442C-AEDANS is situated at the base of the active site. T554C-AEDANS is the residue adjacent to Glu-553, and both of these residues are located on a β -strand that forms part of the active site, with the side chain of Glu-553 protruding into the active-site cleft and the side chain of Thr-554 pointing away from the active site. Glu-553 was shown to be an active-site residue through photoaffinity labelling experiments, where irradiation of ETA with UV light resulted in the covalent attachment of nicotinamide to Glu-553 [36], and also by site-directed mutagenesis, where the mutant E553D exhibited a 1000-fold reduction in enzyme activity [37]. The crystal structure of the catalytic domain of ETA shows Glu-553 forming an hydrogen bond with the oxygen of the thiazole-ribose of β -TAD (an NAD⁺ analogue in which thiazole replaces nicotinamide). A519C-AEDANS is situated on a loop region that is close to the bound NAD⁺ substrate [approx. 10 Å (1 Å = 0.1 nm) from the nicotinamide]. Addition of the fluorescent probe at position 519 resulted in a 95% loss of enzyme activity (Table 1), illustrating that the probe may interfere with the ability of NAD⁺ to gain access to or to orient properly within the active site. S507C-AEDANS is distant from the active site and if the minimal contact model is valid, this residue would not have been identified probably as a potential contact site. However, acrylamide quenching can only detect the ability of the quencher to access the fluorescent probe. It has been assumed that a reduction in k_q value is a result of contact by eEF2; however, the possibility that eEF2 complexation with the toxin leads to conformational changes induced at sites distant from the actual contact site cannot be eliminated by this technique alone. A conformational change could reduce the accessibility of a site-specific probe although that site did not directly contact the eEF2 protein. Complexation of proteins often involves the movement of surface loops or short segments of the protein, as well as the rotation of the side-chains from surface-exposed residues [38]. The fluorescence quenching data for S507C-AEDANS may be an example of a change in k_q value that resulted from a conformational change caused by eEF2 binding.

Fortunately, a three-dimensional structure of eEF2 from *Saccharomyces cerevisiae* has recently been solved to 2.9 Å [16]. Although the work presented in that paper was performed using eEF2 isolated from wheat germ, many inferences can be made between the eEF2 proteins from these differing species since sequence conservation is high – for instance, the identity and

Figure 5 Crude model of the PE24H-eEF2 complex

(a) Comparison of the known structure of eEF2 from yeast and the modelled structure of eEF2 from sugar beet. The modelled structure of sugar-beet eEF2 (*Beta vulgaris*) was generated using its primary sequence, the template structure from yeast eEF2 and SWISS-MODEL version 36.0003 [39,40]. The resulting modelled structure of sugar-beet eEF2 had 64.8% identity with the yeast eEF2. (b) Connolly surface of the three-dimensional interactions between PE24H and eEF2 (yeast) showing the electrostatic surface potential. The NAD⁺ analogue, β -TAD, is shown in stick form. Residues shown as shaded space-filled structures within PE24H are potential eEF2 contact sites. The site of toxin attack on eEF2, namely the diphthamide residue, is also shown as a space-filled structure. The positive clusters on eEF2 are shown as **I** (β -strand ⁵⁷⁸KSPNKHN⁵⁸⁵R) and **II** (Lys-632 and Lys-651). The negative clusters on PE24H are shown as **III** (loop region containing Glu-546, Glu-547 and Glu-548) and **IV** (loop containing Asp-484, Glu-486 and Asp-488). The residues are: 1, Ser-507; 2, Thr-554; 3, Ala-519; 4, Ser-459; 5, Ser-449; 6, Thr-442; 7, Glu-486; 8, Ser-410 and 9, Ser-408. The protruding lobe on eEF2 is indicated as **V**. The full co-ordinates of eEF2 from yeast (*S. cerevisiae*) (PDB entry, 1NOV) [16] and the PE24H/ β -TAD complex (PDB entry, 1AER) [8] were obtained from the RCSB Protein Data Bank (<http://www.rcsb.org/pdb/>). The structures were prepared with the desktop PC modelling package, DS Viewer Pro version 5.0 (Accelrys).

homology between the yeast and the human eEF2 are 65 and 77 % respectively. The complete primary sequence for wheat-germ eEF2 has not yet been determined; however, the N-terminal sequence was previously determined to be VKFTAE (A. Rod Merrill, unpublished work). This N-terminal sequence is identical with that of eEF2 from sugar beet. Since sequence conservation is high between all of the eEF2 proteins, it is reasonable to assume that the sequences of these two plant eEF2 proteins will be highly similar, especially in the structure-determining regions of the sequences. Therefore a modelled structure for sugar-beet eEF2 was generated using the primary sequence of this eEF2 protein and the known structure of yeast eEF2 as the template (Figure 5a); the structures are highly similar with 64.8 % identity. Therefore it was a reasonable supposition to make correlations between wheat-germ eEF2 and yeast eEF2. The dimensions of yeast eEF2 are $122 \times 63 \times 57 \text{ \AA}^3$, illustrating that it is a highly elongated structure. The diphthamide residue (at position 699) is situated at the tip of domain IV making it highly accessible for attack and ribosylation by the toxin enzyme. Based on the elongated structure of eEF2 and exposure of the diphthamide residue, eEF2 probably binds to the catalytic domain of ETA with minimal contact and this model is supported by the fluorescence data herein. Thus a crude contact model for the eEF2–PE24H complex was constructed based on the available protein X-ray structures along with the fluorescence data in the present study, while paying careful attention to possible electrostatic interactions between the two proteins. Protein–protein interfaces consist largely of polar and charged residues, and electrostatic networks and hydrogen bonds help to stabilize protein complexes [24]. Within the eEF2 structure, two separate positively charged patches exist on each side of the diphthamide residue. The predominant positive cluster is found as part of a β -sheet structure containing, ⁵⁷⁸KSPNKH⁵⁸⁵R. The other positive cluster is formed from Lys-632 and Lys-651. On PE24H, two corresponding negative patches can be seen. One negative patch is formed near the active site by a loop region consisting of three glutamate residues (Glu-546, Glu-547 and Glu-548), whereas the other is created in part by the catalytic loop from Asp-484, Glu-486 and Asp-488. The complimentary electrostatic clusters (positive in eEF2 and negative in PE24H) between eEF2 and PE24H were aligned and the diphthamide was positioned near the scissile N-glycosidic bond of NAD⁺ (Figure 5b). The alignment of these two proteins in the model has also taken into account the large changes in k_q value observed for S408C- and S410C-AEDANS. During toxin–eEF2 association, since the diphthamide is positioned within the active site, a protruding lobed region on eEF2 (see Figure 5b) may come in close proximity to the fluorescently labelled serine residues at positions 408 and 410 on the toxin. This proposed interaction, combined with the possible conformational effects induced by the catalytic loop, may help to explain the k_q values observed for S408C- and S410C-AEDANS (Table 3).

In summary, a fluorescence-based approach using a series of site-specific fluorescent probes was used to identify any potential contact sites between eEF2 and the catalytic domain of ETA. The fluorescence results have led to the construction of a working model for this protein–protein interaction complex that may serve as a springboard for further investigation concerning the specific nature of the structure and dynamics of the interaction between these two proteins. Comparison of the fluorescence parameters, $\lambda_{em,max}$ and τ_0 , in the presence and absence of eEF2, gave support to the idea that the protein–protein interaction between eEF2 and PE24H does not involve a large number of molecular contacts. Furthermore, a more detailed visualization of this interaction was achieved with acrylamide quenching experiments in which residues that showed a reduction in the solvent accessibility of

the AEDANS probe after association of eEF2 were identified. With the recent determination of the three-dimensional structure of yeast eEF2, this provided the impetus to construct a crude contact model for the eEF2–PE24H complex, which now affords new insight concerning the nature of the interaction between these two proteins. This working model exemplifies the concept that the interaction between these proteins is probably minimal. Owing to the paucity of a high-resolution X-ray or NMR structure of the toxin–eEF2 complex, further work has been initiated, in which a more detailed map of the contact sites will be constructed based on data from fluorescence resonance energy transfer measurements involving specifically labelled intermolecular donor and acceptor sites within both proteins.

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