A Catalytic Loop within *Pseudomonas aeruginosa* Exotoxin A Modulates Its Transferase Activity*

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Ribosyltransferase Motif of P. aeruginosa Exotoxin A

EXPERIMENTAL PROCEDURES

Purification of PE24H—The catalytic fragment of ETA with a C-terminal polyhistidine tag (PE24H) encoded by the plasmid pPH1 was overexpressed in Escherichia coli strain BB101 (DE3) and purified as described (18) with slight modifications. The cells were lysed in an osmotic shock lysate solution (19) containing 10 mM EDTA. The osmotic shock lysate was loaded onto a 10-ml Q-Sepharose Fast Flow anion-exchange column (Amersham Pharmacia Biotech, Baie D’Urfe, Quebec, Canada). The Q-Sepharose column was washed with 50 mM NaCl and 20 mM Tris-Cl (pH 7.9), and the protein was eluted using 300 mM NaCl and 20 mM Tris-Cl (pH 7.9). This eluant was loaded onto a 1-ml chelate-agarose affinity column (Amersham Pharmacia Biotech) charged with 50 mM NiSO₄, as previously described (18), except for the purification of the S55SC and S55SC-pG (where pG is polyglycine)-Loop C mutant proteins, which required the use of 100 mM ZnSO₄ to charge the column. Fractions containing purified protein were pooled and dialyzed against 1 mM EDTA, 50 mM NaCl, and 20 mM Tris-Cl (pH 7.9) and subsequently concentrated to 1 ml using a Centriprep-30 concentrator (Amicon Inc., Beverly, MA).

Purification of eEF-2—eEF-2 was purified from wheat germ as previously described (20) with some modifications.

Site-directed Mutagenesis—The pG-Loop N mutation was created using the Kunkel mutagenesis method as described (21). Two-stage QuikChange™ mutagenesis was used to create the polyglycine Loop C using the Kunkel mutagenesis method as described (21). Two-stage QuikChange™ mutagenesis was used to create the polyglycine Loop C using the Kunkel mutagenesis method as described (21). Two-stage QuikChange™ mutagenesis was used to create the polyglycine Loop C using the Kunkel mutagenesis method as described (21).

Fluorescence Measurements—All steady-state fluorescence measurements (except for the eEF-2 binding assay) were obtained using a PTI fluorometer equipped with a cooled photomultiplier tube and a water-jacketed sample chamber set to 25 °C.

ADPRT Assay (NAD⁺-dependent)—The ADPRT activity of the various enzyme samples was tested as described previously (20). Briefly, the excitation and emission monochromators were set to 305 nm and zero order (no diffraction), respectively, and a 309-nm cutoff filter (Oriel Corp., Stratford, CT) was included on the sample chamber side of the excitation and emission monochromators. Buffer (20 mM Tris-Cl (pH 7.9)), e-NAD⁺ (Sigma; various concentrations were used up to 500 μM from an e-NAD⁺ stock solution prepared in distilled water, ε₂₆₅nm = 6000 M⁻¹ cm⁻¹), and 14 μM eEF-2 (at saturating levels) were combined in an ultramicrocuvette (3-mm path length; Helma Inc., Concord, Ontario, Canada). The cuvette was equilibrated at 25 °C for 10 min. Toxin dilutions were performed in siliconized tubes with the buffer described above. The reaction was started by rapidly adding PE24H (final concentrations for tox ranged from 5 nM to 19 μM depending upon the mutant protein), and the reaction progress was monitored by an increase in fluorescence during the production of e-ADP-ribose.

ADPRT Assay (eEF-2-dependent)—The assay was performed as described above with the following modifications. Necessary adjustments to the eEF-2 protein solution were made to maintain KCl concentrations at 85 mM since it was stored in 300 mM KCl-containing buffer.
Assay conditions were established whereby e-NAD⁺ remained constant (500 μM representing saturation) and the concentration of eEF-2 varied (1.5–15 μM). Toxin concentrations ranged from 10 nM to 8.8 μM (final concentration).

**ε-AMP Standard Curve and Assay Calibration**—A stock solution of ε-AMP (Sigma; prepared in distilled water; ε265nm = 10,000 M⁻¹ cm⁻¹) was used to prepare a series of standards in 20 mM Tris-HCl (pH 7.9). The fluorescence of the ε-AMP standards (0–10 μM) was recorded to generate a standard curve having a slope with units of fluorescence intensity per micromolar ε-AMP. This slope from the standard curve was used to convert the slopes obtained for the ADPRT measurements to catalytic rates with units of micromolar.

Quenching of Intrinsic Protein Fluorescence—The NAD⁺-dependent quenching of the intrinsic protein fluorescence of the tryptophans in PE24H was used to determine the binding constant (Kₒ) for NAD⁺ and was observed by exciting the protein at 295 nm (4-nm slit width) and measuring the fluorescence intensity with the emission wavelength set to 340 nm (4-nm band pass) as previously described (6). Briefly, the protein solution was titrated with concentrations of NAD⁺ ranging from 0 to 981.5 μM in the presence of toxin in an initial volume of 600 μl in 50 mM NaCl and 20 mM Tris-HCl (pH 7.9). The final concentration of toxin used in this experiment was 1.25 μM, except for D484A/D488A and Q483A/D486A/D488A, which were at 0.625 μM.

**Fluorescence Labeling of PE24H**—Purified PE24H protein (0.5 mg of protein) was incubated in 150 mM NaCl and 200 mM Tris-HCl (pH 8.1) with an excess of DT (5 mol of DT/mol of PE24H) at room temperature for 60 min. A concentrated solution of IAEEDANS (in MeSO) was added to the reaction mixture (5% MeSO, final concentration) to give a molar ratio of 20.1 IAEEDANS/PE24H, and the reaction was gently mixed on a nutator for 15 min at room temperature. The reaction was quenched by the addition of an excess of DT (100 μM final concentration). The quenched sample was loaded onto a prepacked EconoPac 10DG column (Bio-Rad, Mississauga, Ontario), and the protein adduct was eluted with 150 mM NaCl and 20 mM Tris-HCl (pH 7.9). The protein adduct peak was well separated from the free dye peak, with the latter peak appearing in later fractions. The labeled PE24H protein was further characterized by UV and fluorescence spectroscopy using the molar extinction coefficient for 5-AF (ε295nm = 27,510 M⁻¹ cm⁻¹) and for AEDANS (ε295nm = 6000 M⁻¹ cm⁻¹) (24). The labeling efficiency, based on the appropriate absorbances and molar extinction coefficients of the bound fluorophore and the measured protein, was nearly 100% (1 mol of AEDANS/mol of PE24H).

**Fluorescence Labeling of eEF-2**—Purified wheat germ eEF-2 protein was labeled with 5-iodoacetamide fluorescein (5-IAF, Molecular Probes, Inc.) or 5-iodoacetamide rhodamine (5-IAR, Molecular Probes, Inc.) by incubation of 1 mg of protein in reaction buffer (200 mM Tris-HCl (pH 8.1) containing DT (3 mol of DT/mol of protein) for 30 min, followed by the addition of a concentrated stock of 5-IAF (20 mg/ml in MeSO at 10 mol of 5-IAF/mol of protein). The reaction solution was gently mixed on a nutator, and the sulfhydryl labeling reaction was stopped after 10 min at 25 °C by the addition of a 100-fold excess of DT. The reaction mixture (415 μl) was loaded onto a 10DG column (Bio-Rad, Mississauga, Ontario), and the protein adduct was eluted with 50 mM NaCl and 20 mM Tris-HCl (pH 7.9), which was observed by exciting the protein at 295 nm (4-nm slit width) and measuring the fluorescence intensity with the emission wavelength set to 325 to 355 nm in ultramicrocuvettes. The excitation and emission slit widths were both 4 nm. Unfolding profiles were determined as previously described (21, 24).

**RESULTS**

**Location and Nomenclature of the Loop Regions**—The sequences of two loop regions within the catalytic domain of ETA with proposed involvement either in catalysis or through an association with eEF-2 are shown and aligned with the corresponding sequences within DT (Fig. 1). The nomenclature (ETA numbering) for these loops is such that the loop closest to the N terminus of the protein is termed Loop N (Arg458-Ala469), and the loop closest to the C terminus is Loop C (Gln483–Arg490). The alignment of these two loops within ETA did not produce a good match between Loop N and the corresponding loop within DT; however, the alignment of Loop C showed good alignment (sequence identity and homology) with the corresponding loop within DT (Fig. 1). Notably, there is sequence conservation at Asp484 and Glu486 within the Loop C region of these two diphthamide-specific toxins. Interestingly, Loop C did not appear in the original crystal structure of intact whole ETA (25); however, it was resolved in the structure of the catalytic domain of ETA (8, 9). The three-dimensional structure of Loop N was not fully resolved in the catalytic domain structure that bound a less hydrolyzable NAD⁺ analog, β-methylene-thiazole-4-carboxylic adenine dinucleotide (β-TAD), and it was discovered (9), but it was evident in the structure of whole ETA and the catalytic domain complexed with nicotinamide and AMP (8). It has been shown that, upon proteolytic cleavage within a helix in the translocation domain of the whole toxin, Loop N moves to allow greater access to the active site; and consequently, it has been proposed to interact with eEF-2 due to its solvent exposure upon shifting (9).

**Polyglycine Loop Replacement Investigation**—One possibility
in the study of the importance of either Loop N or C would be to delete the entire region. However, analysis of the crystal structure (9) showed that this might not be advisable. The distance between the beginning and end of the loop is significant, and its removal would impose tight constraints on the protein structure, thereby altering it. The distance between the beginning and end of Loop C was determined to be 14.6 Å (between the C-α atoms of Gin482 and Arg499), and this distance is 12.4 Å (between the C-α atoms of Ala457 and Ala464) for Loop N. An alternative to loop deletion mutants was to replace each loop with a glycine segment. Glycine is a very unreactive residue that allows much flexibility in terms of possible main chain conformations. Therefore, in the alternative approach, the loop region is replaced with a glycine sequence. The data in Table II indicate that only polyglycine mutants are shown in Table II and were constructed in the catalytic domain of ETA (PE24H) (18), and their importance was initially screened by their ADPRT activity to determine if either or both of these regions were involved in catalysis. The kinetic parameters for NAD⁺ as substrate for these polyglycine mutants are shown in Table II and were determined with eEF-2 at saturating levels (14 μM). The pG-Loop N mutant protein had a lower activity than the wild-type protein (18,000-fold decrease) when both loops are replaced with glycine was further attenuated; however, this decrease was not strictly cumulative. In essence, the major contributing region affecting catalysis is Loop C, with Loop N playing a minor role.

An investigation into the structural integrity of the loop mutants was undertaken. pG-Loops N and C are expressed as soluble proteins at levels similar to those of the wild-type protein. The sensitivity of these mutant proteins to trypsin proteolysis compared with the wild-type protein was also investigated. Examination of the resultant proteolytic pattern and time required for complete digestion indicated that all three loop replacement mutants (pG-Loop N, pG-Loop C, and DLM) were slightly less stable, but were structurally similar to the wild-type protein (data not shown). Furthermore, the chemical unfolding of pG-Loop C was studied using urea as the denaturant; the data were fit to a two-state folding model as previously described (21); and the results for the free energy change associated with this unfolding (∆G(U→D)) and the transition midpoints (D0) were estimated (data not shown). ∆G(U→D) represents the free energy change for the conversion of the native to unfolded state of a protein in the absence of denaturant. Comparison of the values of ∆G(U→D) for the wild-type and pG-Loop C proteins showed that less free energy was needed to unfold the mutant protein. ∆G(U→D) for the wild-type protein is 8.8 ± 2.9 kJ mol⁻¹, whereas that for pG-Loop C is 5.5 ± 2.5 kJ mol⁻¹. This instability at lower urea concentrations is reflected in the transition midpoints, which were 1.1 and 1.7 M for the mutant and wild-type proteins, respectively. The instability of the mutant protein at lower concentrations of urea may be a reflection of some instability in the region of the loop; however, as the protein was titrated with urea, unfolding like that of the wild-type protein was observed. Additional support for pG-Loop C having a similar structure as the wild-type protein is given by the fluorescence wavelength emission maximum (fluorescence λ_em(max)). In the absence of denaturant, both of these proteins have a fluorescence λ_em(max) at 334 nm, illustrating that initially the tryptophan residues in these proteins are in a similar environment and therefore similarly localized in the tertiary structure of the proteins. For the fully denatured proteins, the fluorescence λ_em(max) is 350 nm. Since the three tryptophans are situated within or near the active site of the enzyme, these data suggest that the active site in this mutant protein has a similar structure to that in the wild-type protein.

Further evidence for the native-like fold of the pG-Loop C mutant protein is shown in Fig. 3, for which an assay was devised for the binding of toxin with its eEF-2 substrate based on fluorescence resonance energy transfer between donor-labeled PE24H (AEDANS) and acceptor-labeled eEF-2 (5-AP). The binding data were similar for the pG-Loop C mutant compared with the wild-type protein (K_D = 1526 ± 76 and 1471 ±

### Table II

| Relative k_cat | Relative K_m(NAD⁺) | Relative specificity constant (k_cat/K_m) |
|---------------|-------------------|----------------------------------------|
| Wild-type     | 1.00 ± 0.02       | 1.00                                   |
| pG-Loop N     | 0.056 ± 0.001     | 0.29 ± 0.02                            |
| pG-Loop C     | (5.6 ± (9.0) × 10⁻³) | 0.21 ± 0.02 | 2.8 × 10⁻⁴ |
| DLM           | ND                | ND                                     |
| Q483A         | 0.072 ± 0.002     | 1.39 ± 0.05 | 0.05 |
| D484A         | 0.039 ± 0.005     | 0.93 ± 0.03 | 0.04 |
| Q485A         | 0.189 ± 0.030     | 0.97 ± 0.25 | 0.18 |
| E486A         | 0.653 ± 0.024     | 1.81 ± 0.16 | 0.34 |
| P487A         | 0.320 ± 0.011     | 1.16 ± 0.04 | 0.27 |
| D488A         | 0.077 ± 0.001     | 1.27 ± 0.09 | 0.06 |
| R490A         | 0.907 ± 0.039     | 1.36 ± 0.04 | 0.67 |
| D484A/D488A   | 0.003 ± 0.001     | 0.52 ± 0.06 | 0.006 |
| Q483A/D484A   | 0.002 ± 0.0001    | 1.24 ± 0.06 | 0.006 |

- The wild-type k_cat and K_m values were 748 ± 16 min⁻¹ and 307 ± 24 μM, respectively.
- The wild-type k_cat and K_m values were 878 ± 27 min⁻¹ and 179 ± 47 μM, respectively.
- The wild-type k_cat and K_m values were 836 ± 22 min⁻¹ and 269 ± 21 μM, respectively.

The transition state species for the ADPRT reaction is likely more affected than the ability to bind the substrate, NAD⁺ binding data for the two polyglycine loop mutants confirmed that NAD⁺ bound normally to both mutant proteins (Fig. 2), with K_D values of 215 ± 16 and 191 ± 19 μM for the pG-Loop N and pG-Loop C mutants, respectively.

DLM (both loops replaced with glycine) was also assessed; however, because its activity was at base-line levels, reliable kinetic parameters could not be obtained. As an alternative, the rate of reaction at an NAD⁺ concentration of 200 μM was used to compare the mutant and wild-type proteins. This comparison indicated that the turnover number is in the range of 65,000-fold less than that of the wild-type protein (Table II). However, despite its low activity, DLM also was capable of binding NAD⁺ (149 ± 12 μM) (Fig. 2). Therefore, the effect on catalysis when both loops are replaced with glycine was further attenuated; however, this decrease was not strictly cumulative.

The sensitivity of these mutant proteins to trypsin proteolysis compared with the wild-type protein was also investigated. Examination of the resultant proteolytic pattern and time required for complete digestion indicated that all three loop replacement mutants (pG-Loop N, pG-Loop C, and DLM) were slightly less stable, but were structurally similar to the wild-type protein (data not shown). Furthermore, the chemical unfolding of pG-Loop C was studied using urea as the denaturant; the data were fit to a two-state folding model as previously described (21); and the results for the free energy change associated with this unfolding (∆G(U→D)) and the transition midpoints (D0) were estimated (data not shown). ∆G(U→D) represents the free energy change for the conversion of the native to unfolded state of a protein in the absence of denaturant. Comparison of the values of ∆G(U→D) for the wild-type and pG-Loop C proteins showed that less free energy was needed to unfold the mutant protein. ∆G(U→D) for the wild-type protein is 8.8 ± 2.9 kJ mol⁻¹, whereas that for pG-Loop C is 5.5 ± 2.5 kJ mol⁻¹. This instability at lower urea concentrations is reflected in the transition midpoints, which were 1.1 and 1.7 M for the mutant and wild-type proteins, respectively. The instability of the mutant protein at lower concentrations of urea may be a reflection of some instability in the region of the loop; however, as the protein was titrated with urea, unfolding like that of the wild-type protein was observed. Additional support for pG-Loop C having a similar structure as the wild-type protein is given by the fluorescence wavelength emission maximum (fluorescence λ_em(max)). In the absence of denaturant, both of these proteins have a fluorescence λ_em(max) at 334 nm, illustrating that initially the tryptophan residues in these proteins are in a similar environment and therefore similarly localized in the tertiary structure of the proteins. For the fully denatured proteins, the fluorescence λ_em(max) is 350 nm. Since the three tryptophans are situated within or near the active site of the enzyme, these data suggest that the active site in this mutant protein has a similar structure to that in the wild-type protein.

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The binding constants for the polyglycine loop mutants with the NAD$^+$ substrate were similar to the wild-type protein (see text). The pG-Loop C mutant protein showed a modest increase in the $K_D$ value compared with the wild-type protein (nearly 4-fold). These data indicate that the polyglycine loop mutant proteins show near wild-type substrate-binding properties.

Alanine-scanning Mutagenesis—The role of each residue within Loop C was probed using alanine-scanning mutagenesis based on the results obtained from the study of the polyglycine replacement mutant proteins (17). Like the earlier study involving the polyglycine mutant proteins, these alanine mutant proteins were assessed for their ADPRT activity. As shown in Table II, none of the single alanine replacement mutant proteins displayed a significant alteration in $K_m$ for the NAD$^+$ substrate compared with the wild-type protein. However, three mutant proteins had reduced $k_{cat}$ values. The activities of the mutant proteins Q483A, D484A, and D488A were 7.2, 4.0, and 7.7%, respectively, of that of the wild-type protein. Table III indicates that the dissociation constants for NAD$^+$ binding were not adversely affected by the alanine replacements. Although D484A and D488A did show a decreased affinity for this substrate (2.8- and 2.4-fold, respectively), the effect was not large in comparison with the observed effect on the $k_{cat}$ values for these mutant proteins. Therefore, these data indicate that the NAD$^+$ substrate-binding site was not impaired by any of the alanine replacements within Loop C. Trypsin proteolysis studies on the three alanine mutant proteins that exhibited the most reduced ADPRT activity (Q483A, D484A, and D488A) showed band patterns and time courses similar to those of the wild-type protein (data not shown). Therefore, to resolve whether or not the cause of the defect in Loop C was related to an alteration in the eEF-2 substrate site, the kinetic parameters for the eEF-2 substrate were determined (variable eEF-2 concentrations and [e-NAD$^+$] fixed at 500 μM) (Table IV) on those alanine mutant proteins that had previously showed altered ADPRT activity.

76 nM, respectively). The wild-type protein is actually represented by a catalytically innocuous single Cys mutant (S585C) that has normal ADPRT enzymatic activity.
As dictated by the turnover numbers (Table IV), Q483A, D484A, and D488A had significantly reduced activity. The $K_m$ values for these alanine mutant proteins were only marginally increased (1.02–2.19-fold), indicating that Loop C plays a major role in the catalytic process within ETA and not in substrate association. The pG-Loop C protein was also examined; however, its activity was only marginally above background in this assay (eEF-2 as limiting substrate); and therefore, a $K_m$ for eEF-2 for this loop mutant could not be determined. It was noted that the magnitudes of the effects on the relative $k_{cat}$ values showed some differences, as shown in Tables II and IV (relative $k_{cat(eEF-2)}$ and $k_{cat(eEF-2)}$ values) as follows: Q483A, 0.07 and 0.01; Q484A, 0.04 and 0.07; Q485A, 0.19 and 0.69; and D488A, 0.08 and 0.17, respectively. However, these differences are not large and likely reflect the use of separate batches of the protein substrate (eEF-2) for each data set. Notably, although the magnitudes of the changes in the $k_{cat}$ values showed some differences, the trends remained consistent.

Two additional mutant proteins (multiple replacements) were made based on the initial activity measurements of the alanine mutant proteins. These were D484A/D488A and Q483A/D484A/D488A, which were used to determine if these residues are primarily responsible for the reduced catalysis of the entire loop region. Both D484A/D488A and Q483A/D484A/D488A had reduced activity (relative $k_{cat}$ values for both were <0.5% of the wild-type value), with a minimal effect on $K_m$ (eNAD$^+$) (Table II). It is notable that when both aspartate residues were mutated to alanine, the resulting $k_{cat}$ equaled the accumulation of the individual alanine mutant, suggesting that Asp$^{484}$ and Asp$^{488}$ cooperate in an event linked to catalysis. However, the addition of the mutation Q483A to the D484A/D488A mutant (Q483A/D484A/D488A) did not further significantly reduce the $k_{cat}$. Therefore, not all three residues act in concert. The NAD$^+$ binding studies on D484A/D488A and Q483A/D484A/D488A showed near wild-type protein behavior (Table III), providing further support for the idea that Loop C participates in an important event in catalysis.

**TABLE IV**

Comparison of ADPRT activity upon varying the eEF-2 substrate for the alanine-scanning mutants of Loop C

| Protein                  | $K_m$ $^a$ | $k_{\text{cat(eEF-2)}}$ $^a$ | $K_m$ $^{\text{em(max)}}$ $^b$ |
|--------------------------|------------|-------------------------------|----------------------------------|
| Wild-type                | 55 ± 1     | 1.00 ± 0.12                   | 1.00 ± 0.07                       |
| Q483A                    | 77 ± 9 (1.4) | 1.02 ± 0.09                   | 1.11 ± 0.01                       |
| D484A                    | 156 ± 15 (2.8) | 2.19 ± 0.21                   | 0.03 ± 0.02                       |
| Q485A                    | 50 ± 3 (0.9) | 1.01 ± 0.05                   | 0.68 ± 0.02                       |
| D488A                    | 76 ± 8 (1.4) | 1.05 ± 0.01                   | 0.08 ± 0.01                       |
| R490A                    | 131 ± 4 (2.4) | 2.06 ± 0.13                   | 0.08 ± 0.01                       |
| D484A/D488A              | 73 ± 6 (1.3) |                               |                                  |
| Q483A/D484A/D488A        | 98 ± 10 (1.8) |                               |                                  |

$^a$ Values represent the mean ± S.D. of experiments performed in triplicate, except for D484A/D488A and Q483A/D484A/D488A, which were in duplicate.

$^b$ Numbers in parentheses represent the -fold increase in $K_m$ compared with wild-type PE24H.

**DISCUSSION**

In the initial polyglycine loop investigation, pG-Loop C had a significant loss of activity (18,000-fold decrease), yet was able to retain its ability to interact with both NAD$^+$ and eEF-2 substrates. However, the structural integrity of this mutant became in question because of this activity defect. As was shown, the NAD$^+$ binding data illustrated that pG-Loop C is capable of binding NAD$^+$ with a similar affinity as the wild-type protein. Hence, the pG-Loop C mutant protein has a folded structure with an active site resembling that of the wild-type protein. Earlier work has shown that $N$-acetyltryptophanamide, a control for free tryptophan, does not bind NAD$^+$ since no tryptophan quenching was observed (6). Therefore, it is unlikely that the quenching of the intrinsic protein fluorescence from this mutant protein would be observed if the protein was significantly unfolded. Moreover, it has been demonstrated that NAD$^+$ quenching of tryptophan fluorescence is protein-specific (20). Furthermore, the tryptophan fluorescence $\Delta_{\text{em(max)}}$ for pG-Loop C is identical to that for the wild-type protein, indicating that these proteins are folded properly. Chemical denaturation (although suggesting slightly reduced stability), combined with the proteolysis study, demonstrated that pG-Loop C is not misfolded and has a similar structure as the wild-type protein. Therefore, the experimental data indicate that this loss of activity for pG-Loop C must correlate with the replacement of critical residue(s) involved in the ADP-ribosyltransferase step within the catalytic mechanism of the toxin enzyme. Unfortunately, this study alone could not fully decipher the significance of Loop C. As an extension of this work, alanine-scanning mutagenesis on Loop C was used to determine if specific residues within the loop or if an inherent feature of the loop is responsible for what was observed in the pG-Loop C study.

The other loop region in this study, Loop N, does not appear to be an essential catalytic factor since its replacement with polyglycine resulted in a significant, but relatively small decrease in enzymatic activity. This loop region was previously investigated when the crystal structure for the catalytic domain of ETA was solved in the presence of an NAD$^+$ analog. Results showed that the binding of NAD$^+$ does not stabilize this loop, indicating that this region is not needed for NAD$^+$ binding (8, 9). Li et al. (8, 9) also proposed that this loop may be able to interact with eEF-2 since it becomes solvent-accessible once the toxin is activated and cleaved. This process shifts the loop from protecting the active site in the proenzyme state to a conformation that exposes the active site. When compared with other toxins, this Loop N region shows no structural similarities. The corresponding loop region in diphtheria toxin, which also ADP-ribosylates eEF-2, is much longer. The x-ray structures of both catalytic domains of ETA and DT superimpose (9, 25, 26); therefore, if a catalytic motif exists within this enzyme, it is likely not contained within this region.

The kinetics of the alanine replacement mutant proteins within Loop C suggested the identity of a small subregion within this loop that is responsible for the decrease in ADPRT activity, namely Glu$^{482}$–Asp$^{484}$ and Asp$^{488}$. Since NAD$^+$ and eEF-2 binding data and the $K_m$ values are similar to those of the wild-type protein, it suggests that Loop C is an important catalytic element within ETA. In the structure of the catalytic domain of ETA, the phenol ring of Tyr$^{481}$ stacks with the nicotinamide ring of NAD$^+$ (8, 9) near the site of cleavage where the ADP-ribosyl group of NAD$^+$ is transferred to eEF-2. Therefore, residues involved in this transfer event must be spatially situated near Tyr$^{481}$. Examination of
the position of the subregion (in particular, Gln\(^{483}\), Asp\(^{484}\), and Gln\(^{485}\)) (Fig. 4) correlates with this notion since they are on one face of the loop in close proximity to Tyr\(^{481}\) and are located close to the NAD\(^+\) substrate. However, the remaining residues within Loop C are situated on the opposite side of the loop and are more distant from the site of the reaction. Asp\(^{488}\) is important for activity; however, it is not situated as closely to Tyr\(^{481}\) as the other catalytically important residues in question. However, the kinetic data for the D484A/D488A mutant enzyme showed that Asp\(^{488}\) acts in concert with Asp\(^{484}\). Unfortunately, the distance between these two residues is too large for any direct interaction, but these residues could be linked through a bridged water molecule (however, it was not resolved as a heteroatom in the structure (9)) since the x-ray structure shows Asp\(^{488}\) participating in several hydrogen bonds. Therefore, Asp\(^{488}\) may play a structural role within the loop region by properly aligning those residues (in particular, Gln\(^{483}\), Asp\(^{484}\), and Gln\(^{485}\)) that are perhaps involved in the stabilization of the transition state for the ADPRT reaction, which would involve parts of both the NAD\(^+\) and eEF-2 substrates as the kinetic data suggest (Tables II and IV).

To investigate if a possible ADP-ribosyltransferase motif of the eEF-2-specific ADP-ribosylation reaction had been uncovered, a comparison of the catalytic domains of ETA and DT was undertaken. Both of these proteins recognize eEF-2 and have structurally similar active sites (8, 25, 26). The structure of the NAD\(^+\)-bound catalytic domain of DT was superimposed with the catalytic domain of ETA, and a sequence alignment was proposed (25). The alignment illustrated that Tyr\(^{470}\) and Tyr\(^{470}\) in ETA correspond to Tyr\(^{65}\) and Tyr\(^{54}\) in DT, respectively, in which the nicotinamide ring is positioned within a groove created by these tyrosines. In addition, those residues in DT that align with Loop C from ETA form a loop with notable sequence identity and residue conservation. Importantly, Asp\(^{484}\), shown by the alanine-scanning experiment as the most critical catalytic residue, is conserved between these two diphthamide-specific toxins. At Gln\(^{485}\) (a residue important for catalysis) in Loop C, a conservative substitution of Asn\(^{69}\) in DT is found. The E486A mutation within Loop C gave no indication that this glutamate residue is necessary for catalysis; however, it is conserved within DT (Glu\(^{70}\)) (Fig. 1). However, analysis of the entire Loop C region (Val\(^{67}\)–Ser\(^{74}\)) shows that Gln\(^{70}\) in DT may be required to maintain the overall net charge of this catalytic motif. As discussed, Gln\(^{483}\) and Gln\(^{485}\), in addition to Asp\(^{484}\), represent the region proposed to stabilize the transition state during the transferase step. These residues are polar and have the ability to hydrogen bond. The residues analogous to the glutamines in DT are Val\(^{67}\) and Asn\(^{69}\), with only the latter having this property. Although a hydrophobic residue corresponds to Gln\(^{483}\) in the sequence alignment, a serine residue positioned next to this valine may participate in the hydrogen bonding in the DT structure. The remaining residues in Loop C of ETA have little similarity to the corresponding residues in DT and are located on the opposite face of the loop away from the active site (Fig. 4). Asp\(^{488}\) in Loop C is proposed to play a role in stabilizing Loop C; yet in DT, at this position is found a proline, which cannot stabilize the loop through hydrogen bond formation. Therefore, other residues in DT must be responsible for stabilization of the active-site loop structure. In conclusion, an active-site structure exists within DT that is analogous to the predicted ADP-ribosyltransferase structure within Loop C and is also near the site of ADP-ribose transfer (Fig. 4 and Tables II and IV). It is proposed that this catalytic region within these two diphthamide-specific ADPRT enzymes represents a catalytic structural motif that appears to be specific to only this subclass of the ADPRT family (27).

In summary, a working model is proposed that illustrates how these data may relate to catalysis. Initially, a binary complex between ETA and NAD\(^+\) forms (14, 28), exposing the transferase site. The eEF-2 substrate then docks onto the surface of the enzyme, and the transition state structure is stabilized by favorable interaction with Loop C (Gln\(^{483}\), Asp\(^{484}\), and Gln\(^{485}\)) within the catalytic domain of the toxin. This alignment and stabilization of the transition state structure within the active site by the catalytic residues Gln\(^{483}\), Asp\(^{484}\), and Gln\(^{485}\) may occur through the formation of hydrogen bonds. The completion of the transferase event then induces a conformational change within the complex, weakening the association between eEF-2 and the enzyme, thereby releasing ADP-riboyl-eEF-2.

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Fig. 4. Proposed ADP-ribosyltransferase motif within Loop C of P. aeruginosa exotoxin A (A) and diphtheria toxin (B). The residues within the proposed motif are shown as ball and sticks. The NAD\(^+\) and β-TAD\(^+\) (β-TAD\(^+\)) substrates are shown in purple, and the active-site tyrosine residues (Tyr\(^{470}\) and Tyr\(^{481}\) in ETA and Tyr\(^{54}\) and Tyr\(^{65}\) in DT) are fuchsia and red, respectively. The residues in Loop C of ETA and the corresponding loop in DT are labeled and shown in color as follows: Gln\(^{483}\) and Val\(^{67}\) (yellow), Asp\(^{484}\) and Asp\(^{488}\) (purple), Glu\(^{69}\) and Asn\(^{69}\) (burgundy), Gln\(^{486}\) and Glu\(^{70}\) (light green), Pro\(^{486}\) and Asn\(^{71}\) (orange), Asp\(^{486}\) and Pro\(^{488}\) (dark green), Ala\(^{492}\) and Leu\(^{72}\) (light blue), and Arg\(^{690}\) and Ser\(^{74}\) (salmon). The rest of the protein structures are shown in gray. The protein structures were created by WebLab Viewer Pro\(^{\text{TM}}\) Version 4.0, and the x-ray structure codes from the Protein Data Bank (www.rcsb.org/pdb) were 1AER (P. aeruginosa exotoxin A) and 1TOX (diphtheria toxin).
Ribosyltransferase Motif of *P. aeruginosa* Exotoxin A

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