# Localization of the Active Site of Diphtheria Toxin†

Jian-Min Zhao and Erwin London*
*Department of Biochemistry, State University of New York at Stony Brook, Stony Brook, New York 11794-5215*
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ABSTRACT: Information about the location of the active site of diphtheria toxin was derived from proteolysis studies and an analysis of its sequence. It was found that a specific trypsin cleavage within whole diphtheria toxin occurs at Lys-39. Therefore, Lys-39 appears to be a surface residue. Furthermore, protection from proteolysis could be obtained upon binding of either the substrate β-nicotinamide adenine dinucleotide (oxidized form) ($NAD^+$) or a competing ligand, adenylyl(3′–5′)uridine 3′-phosphate (ApUp). The protection by ApUp, which binds to the toxin very tightly, required only stoichiometric levels. The most likely explanation of these results is that both $NAD^+$ binding and ApUp binding block trypsin either through a steric mechanism or through a local conformational change, suggesting Lys-39 may be near the active site. Further evidence supporting this conclusion comes from comparison of the previously determined sequences of diphtheria toxin and of *Pseudomonas* exotoxin A, a protein that catalyzes an identical reaction. We find a significant degree of homology between the N-terminal halves of the catalytic domains of these two proteins, which apparently represents active-site residues, and that Lys-39 is in the center of the homologous sequence. Furthermore, the location of the amino acid that is the homologue of Lys-39 within the crystal structure of *Pseudomonas* exotoxin A is also in agreement with a location in or near the active site. Other unusual features in the sequences of diphtheria toxin and *Pseudomonas* exotoxin A are also described, and on the basis of the experiments presented, a possible function for ApUp is considered.

Diphtheria toxin (DT)[1] is a protein produced by *Corynebacterium diphtheriae*. The toxin is secreted as a single polypeptide ($M_r$ 58 348) but can be readily cleaved by proteolysis into two fragments (or subunits), A ($M_r$ 21 167) and B ($M_r$ 37 199), which are joined by a disulfide bond (Pappenheimer, 1977; Collier, 1982; Murphy, 1985) (see Figure 1). The amino acid sequence of the toxin has been determined (Greenfield et al., 1983; Kaczorek et al., 1983; Ratti et al., 1983). Several studies have demonstrated that DT enters cells via receptor-mediated endocytosis and that it then reaches the cytoplasm by penetration through the membrane of an acidic organelle (Draper & Simon, 1980; Sandvig & Olsnes, 1980, 1981; Marnell et al., 1984). The penetration is apparently triggered by a low-pH-induced conformational change in the toxin (Sandvig & Olsnes, 1981; Hu & Holmes, 1984; Zalman & Wisnieski, 1984; Blewitt et al., 1984, 1985). Subsequent to membrane penetration, the A fragment of the toxin is believed to be released into the cytoplasm, where it catalyzes transfer of the ADP-ribose moiety of $NAD^+$ onto the diphthamide residue of elongation factor 2, thereby halting protein synthesis and causing cell death (Pappenheimer, 1977).

Many studies have examined the enzymology of the toxin, and several of these studies have examined its active and ligand binding sites [for a review, see Collier (1982)]. Photolabeling and site-directed mutagenesis studies have identified Glu-148 in fragment A as one residue at the $NAD^+$ binding site (Carroll & Collier, 1984; Tweten et al., 1985). It has also been suggested that Trp-153 is essential because its chemical modification abolishes enzymatic activity (Michel & Dirkx, 1977). Studies of the behavior of the active site have shown $NAD^+$ binding to intact toxin and isolated fragment A is very similar and that both whole toxin and fragment A can catalyze a slow $NAD^+$ glycohydrolase reaction (i.e., breakdown into ADP-ribose plus nicotinamide) (Kandel et al., 1974; Lory et al., 1980a; Collins & Collier, 1984). However, unlike the isolated A fragment, the whole toxin cannot ADP-ribosylate EF-2, perhaps due to steric interference with elongation factor 2 binding (Collier, 1982).

Another unusual feature of ligand association is the extremely tight binding to an endogenous dinucleotide, ApUp (Barbieri et al., 1981; Collins et al., 1984). Both the origin and function of bound ApUp are unclear. Competition studies and structural similarities between ApUp and $NAD^+$ indicate that they bind at strongly overlapping sites (Barbieri et al., 1981; Collins & Collier, 1984). Binding of ApUp may also involve the P site, a site on the B fragment that interacts with phosphorylated ligands (Lory & Collier, 1980a; Proia et al., 1980; Lory et al., 1980b). Most competition and cross-linking studies are consistent with the notion that the P site is spacially adjacent to, and perhaps an extension of, the $NAD^+$ site (Proia et al., 1980; Lory et al., 1980b). Indeed, binding of ApUp to whole toxin is much tighter than binding to fragment A, suggesting some B subunit participation (Collins & Collier, 1984). In addition, a distinguishing characteristic of P-site ligands, such as ATP, is that they prevent binding of toxin to cells (Middlebrook et al., 1978) and under some conditions ApUp can interfere with toxin interaction with cells (Barbieri et al., 1986; Barbieri & Collier, 1987).

In this study, we have examined ligand and substrate protection from proteolysis as an aspect of ligand binding that should provide clues to the location of the active site and

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* Address correspondence to this author.

[1] Abbreviations: DT, diphtheria toxin; PE, *Pseudomonas* exotoxin A; ApUp, adenylyl(3′–5′)uridine 3′-phosphate; $NAD^+$, β-nicotinamide adenine dinucleotide (oxidized form); NADH, β-nicotinamide adenine dinucleotide (reduced form); TLCK, $N^{\alpha}$-(*p*-tosyl)-L-lysine chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; EF-2, elongation factor 2; PTH, phenylthiohydantoin.

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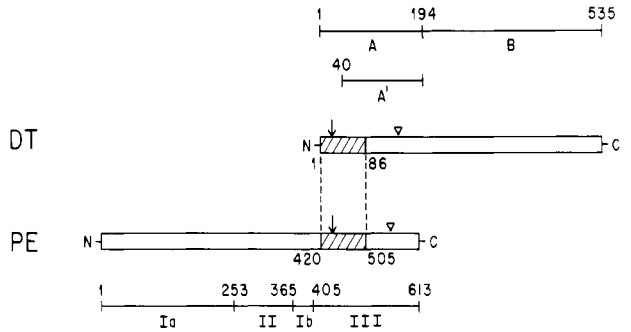


FIGURE 1: Comparison of diphtheria toxin and *Pseudomonas* exotoxin A structures. Domains shown for PE are those derived from the crystal structure. Fragments shown for DT are those described in the text. The hatched area represents the major region of homology. The arrow indicates the position of the conserved His residue. The inverted triangle represents the Glu residue to which $NAD^+$ cross-linking occurs. See text for details.

possible functions for ApUp. We find that there is a proteolysis site on DT which can be protected by binding of either $NAD^+$ or ApUp. We have also identified a previously undetected region of homology between the N-terminal half of the A fragment of DT and the active domain of *Pseudomonas* exotoxin A (PE), another protein toxin that catalyzes $NAD^+$-dependent ADP-ribosylation of the diphthamide residue of elongation factor 2 (Iglewski & Kabat, 1975; Iglewski et al., 1977). The degree of relationship between the two toxins has been unclear. For example, the enzymatically active fragment of PE (Chung & Collier, 1977) is at the C-terminal third of the protein (Gray et al., 1984), whereas fragment A is at the N-terminal side of DT. Furthermore, cross-activity of antibodies to DT or PE to each other is relatively difficult to detect (Sadoff et al., 1982), and when PE was sequenced, no homology with DT was detected (Gray et al., 1984). However, very recently cross-linking has shown Glu-553 in PE is analogous to Glu-148 in DT and that a cluster of a few amino acids in this area shows homology (Carroll & Collier, 1987). In addition, we recently noted that the lone His residue or DT fragment A appears to be in a tetrapeptide conserved in PE (London et al., 1986). A more complete examination now shows a large region of homology between the N-terminal half of the active fragment of PE and that of DT fragment A. Independently, the laboratories of R. J. Collier and D. B. McKay have also discovered this homology (personal communication). Combining information from the proteolysis, homology, and crystal structure of PE (Allured et al., 1986) allows us to derive some tentative conclusions about the active site of DT.

## Experimental Procedures

### *Materials*

Diphtheria toxin was purchased from Connaught Laboratories (Ontario, Canada), and its various forms (Goor, 1968; Barbieri et al., 1980) were purified as described previously (Collier & Kandel, 1971; McKeever & Sarma, 1982). Proteolytic enzymes, ApUp, $NAD^+$, TLCK, 2-mercaptoethylamine hydrochloride, and NADH were purchased from Sigma. [4-$^3$H]$NAD^+$ (1.01 Ci/mmol) was purchased from Amersham.

### *Methods*

*Proteolytic Digestion with Trypsin.* Three micrograms of toxin was digested with 0.15 $\mu$g of trypsin from a freshly prepared solution in a 30 $\mu$L reaction mixture containing 50 mM Tris-HCl, pH 7.2, and 2 mM $CaCl_2$ at 23 °C. After the desired interval, the reaction was stopped by adding SDS to about 1% w/v and 2-mercaptoethanol to 5% v/v. The samples were analyzed on 15% w/v polyacrylamide electrophoresis gels (Laemmli, 1971). When the digestion was carried out in the presence of exogenous ApUp or $NAD^+$, the ligand was added and incubated with toxin for 10 min at 23 °C prior to the addition of protease.

*Preparation of Toxin Fragments.* This was done by a modification of the method of Kandel et al. (1974). Purified bound toxin was treated with trypsin to convert intact to nicked form, as previously described (Blewitt et al., 1984), except that more vigorous conditions were used (25 $\mu$g/mL trypsin; 1.5-h incubation). Trypsin and its inhibitor (TLCK) were removed by gel filtration on HPLC (0.75 × 30 cm, TSK 3000SW equilibrated with 75 mM sodium phosphate, pH 7.0, at a flow rate 0.5 mL/min). Nicked toxin was denatured and reduced in 4 M guanidinium chloride and 25 mM 2-mercaptoethylamine (which after mixing were adjusted to pH 7.0 with $Na_2HPO_4$) for at least 30 min at 23 °C. Toxin fragments were then separated by chromatography on a (1.6 × 95 cm) column of Sephacryl S-200 (Pharmacia), equilibrated with the same buffer. Eluted toxin fragments were dialyzed against 10 mM sodium phosphate, pH 7.0, and concentrated in a vacuum centrifuge (Savant Industries, New York, NY). The fragments were stored at 4 °C. Following trypsin digestion of free toxin, fragment A′ was isolated from HPLC-isolated fragment A′–B complex by using the same procedure.

Toxin concentration was determined by the absorbance as previously described (Blewitt et al., 1985). The concentrations of fragment A and fragment A′ were estimated from the absorbance at 280 nm using an approximate calculated value of $\epsilon_{280} = 23\,100\ M^{-1}\ cm^{-1}$. For the A fragment, this method results in good agreement with those results obtained with the Lowry assay.

*$NAD^+$ Glycohydrolase Assay.* This assay was based on modification of the method of Lory et al. (1980a). The reaction was performed in a volume of 100 $\mu$L containing 50 mM sodium phosphate, pH 7.0–7.2, 50 mM $NAD^+$ (0.5 $\mu$M [$^3$H]$NAD^+$ and 49.5 $\mu$M nonradioactive $NAD^+$), and 100 $\mu$g/mL toxin or toxin fragment. After incubation at 37 °C for the desired time, hydrolyzed [$^3$H]nicotinamide was immediately extracted by vigorous vortexing with 1 mL of water-saturated ethyl acetate. A 0.75-mL aliquot from the upper phase was mixed with Scintiverse (Fisher Scientific) liquid scintillation fluid and radioactivity measured by scintillation counting.

*ApUp and NADH Binding to Toxin.* Titrations of toxin (10 $\mu$g/mL) were carried out in a Spex 212 spectrofluorometer in 2 mL of 50 mM Tris-HCl, pH 7.0 at 23 °C. Aliquots of ApUp or NADH (10–20 $\mu$L) were added to samples containing toxin, and a couple of minutes after each addition fluorescence intensity was measured (excitation wavelength at 280 nm; emission wavelength 330 nm) in a 1-cm path-length quartz cuvette. Correction was made for dilution and inner filter effects (Leese & Wehry, 1978) where necessary.

*Protein Sequencing.* Purified toxin fragments were prepared for sequencing by precipitating with 10% w/v trichloroacetic acid at 0–4 °C, washing 2 times with trichloroacetic acid and 2 times with cold (0 °C) acetone, and then drying. Protein sequencing was performed with an Applied Biosystems (Foster City, CA) gas phase sequencer equipped with an on-line PTH analyzer, using programs supplied by the manufacturer.

## Results

The results of proteolytic attack of various forms of DT by trypsin are shown in Figure 2. The initial attack of trypsin cleaves the toxin into A and B fragments. Free monomer toxin

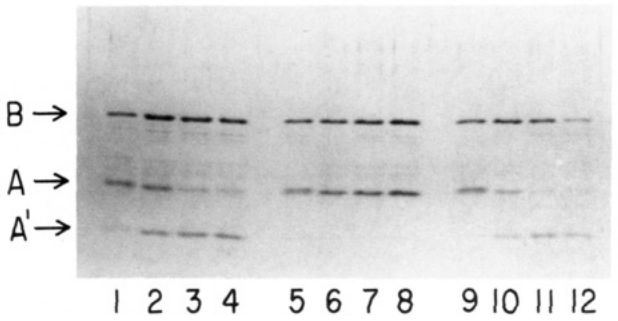


FIGURE 2: Proteolysis of the various forms of toxins by trypsin. Free dimers, lanes 1–4; ApUp-bound dimers, lanes 5–8; free monomers, lanes 9–12. The time of the trypsin digestion was as follows: 0 min in lanes 1, 5, and 9; 30 min in lane 2, 6, and 10; 60 min in lanes 3, 7, and 11; 90 min in lanes 4, 8, and 12. Proteolysis conditions are described under Experimental Procedures.

and free dimer toxin show a further cleavage in which staining intensity indicates that fragment A gradually disappears and is replaced by a smaller fragment. We call the fragment derived from fragment A, A′. The proteolytic degradation of A fragment does not occur with bound toxin, which contains endogenous ApUp. It is digested simply into A and B fragments.

The molecular weight of fragment A′ was estimated to be 17000 from its relative migration on SDS–PAGE by migration relative to standards. Fragment A cleavage should also result in production of a short peptide ($M_r$ about 4000). This peptide, which we have not observed, would be too small to detect by standard SDS gel electrophoresis, and it may be further degraded by trypsin. On nonreducing gels, the A′ fragment is not observed. Instead, the toxin band shows a small decrease in molecular weight, indicating a complex of A′ covalently attached to B is present (not shown). Since the A and B fragments are held together by a disulfide bond which involves Cys-186 near the C-terminal end of the A fragment, this result means that the cleavage which produces A′ must be close to the N-terminus of fragment A (see Figure 1). If instead it was near the C-terminus, nonreducing gels would show two bands, one at the molecular weight of fragment B plus the short peptide and another corresponding to fragment A′. From these results, it appears that fragment A′ corresponds to a trypsin fragment previously identified, although not fully characterized ("fragment D") (Gill & Dinius, 1971).

Fragment A′ was not detected when free toxin was treated with chymotrypsin, with proteinase K, or with papain. Its production by trypsin was not affected by choice of buffer (phosphate or Tris), the presence of 1 mM EDTA, or the presence of 150 mM NaCl (data not shown). However, the amount of fragment A′ produced decreased gradually as pH was decreased, probably due to the intrinsic decrease in trypsin activity at low pH.

In order to confirm ligand presence was responsible for the protection observed with bound toxin, exogenous ligand was added to free monomeric toxin before proteolysis. SDS–PAGE shows that both exogenous ApUp and $NAD^+$ prevent formation of fragment A′ by trypsin (Figure 3). This protection is correlated with the specific binding of these ligands. This is shown by the effect of titration of free toxin with various amounts ApUp or $NAD^+$ (Figure 4). The production of fragment A′ is decreased as the amount of exogenous ApUp or $NAD^+$ is increased in the samples. Protection by ApUp is stoichiometric, complete as soon as its concentration reaches a 1:1 ratio to toxin. Since binding of ApUp to toxin is extremely tight ($K_d$ = 300 pM at 25 °C and 2 nM at 37 °C, Collins et al., 1984), all of the added ApUp will be bound

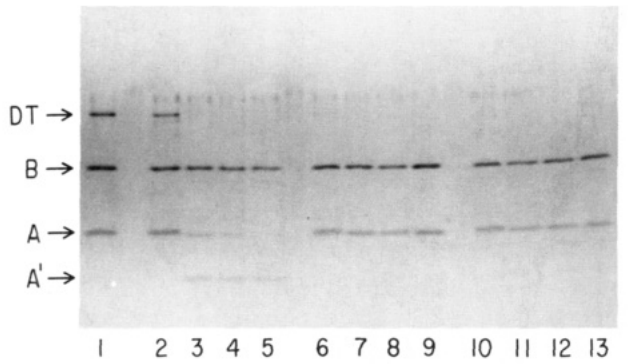


FIGURE 3: Tryptic cleavage of free monomeric toxin in the presence or absence of exogenous ApUp or $NAD^+$. Without ApUp or $NAD^+$ lanes 2–5; with 67 μM ApUp, lanes 6–9; with 0.7 mM $NAD^+$, lanes 10–13. For each set of samples, the time of reaction was 0, 30, 60, and 90 min, respectively, as in Figure 2. The concentration of toxin was 1.72 μM. Lane 1 contains untreated toxin.

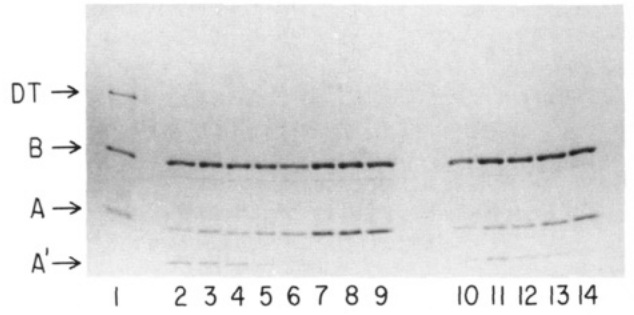


FIGURE 4: Effect of titration of the free monomeric toxin with exogenous ApUp or $NAD^+$ upon tryptic proteolysis. The molar ratio of ApUp to toxin was 0, 0.1, 0.2, 0.5, 1, 2, 5, and 10 (0, 0.17, 0.34, 0.86, 1.72, 3.44, 8.6, and 17.2 μM), respectively, in lanes 2–9. The concentration of $NAD^+$ was 0, 1, 10, 100, and 1000 μM in lanes 10–14, respectively. Samples were digested with trypsin for 1 h. Lane 1 contains untreated toxin.

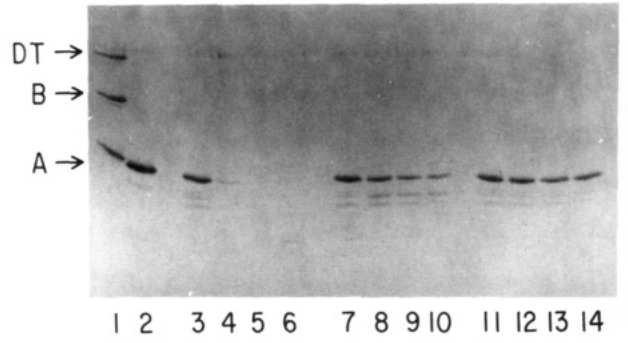


FIGURE 5: Tryptic proteolysis of fragment A in the presence and absence of exogenous ApUp or $NAD^+$. Fragment A without ApUp or $NAD^+$ is shown in lanes 3–6, with 1.16 mM ApUp in lanes 7–10, and with 1.25 mM $NAD^+$ in lanes 11–14. For each set of samples, the time of reaction was 0, 30, 60, and 90 min, respectively, as in Figure 2. The concentration of fragment A was 7.1 μM. Lanes 1 and 2 contain untreated nicked toxin and fragment A, respectively.

under our conditions. Therefore, ApUp protection corresponds exactly with binding. Binding of $NAD^+$ is much weaker ($K_d$ = 10–13 μM at 37 °C, Kandel et al., 1974), and, therefore, it is not surprising that $NAD^+$ protection is not stoichiometric but rather requires much higher concentrations. However, protection is almost complete under conditions in which $NAD^+$ binding is nearly saturating.

$NAD^+$ also protects isolated fragment A from tryptic attack, as briefly noted by Kandel et al. (1974). However, we find there is an important difference between fragment A and whole toxin behavior in this regard. As Figure 5 shows, instead of producing fragment A′, trypsin digestion in the absence of

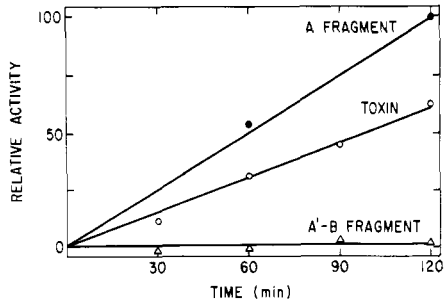


FIGURE 6: $NAD^+$ glycohydrolase activity of toxin. Shown is the time dependence of the amount of [$^3$H]nicotinamide released from $NAD^+$ by fragment A (●), free dimeric toxin (○), and trypsin-treated free dimeric toxin (containing the fragment A'–fragment B complex) (△).

$NAD^+$ seems to degrade fragment A to smaller polypeptides under the same conditions which produce fragment A' when whole toxin is used. Therefore, it is not possible to state conclusively whether protection involves Lys-39 or additional sites in this case. Figure 5 also shows protection of isolated fragment A by ApUp. However, there is an interesting difference in ApUp protection of the A fragment when isolated and in whole toxin. The protection seen in whole toxin at stoichiometric levels of ApUp is lost (data not shown), consistent with the loss of ApUp binding at stoichiometric levels due to its much weaker binding to fragment A ($K_d$ = 140 μM at 37 °C; Collins & Collier, 1984) relative to its binding to whole toxin.

To determine the location of the peptide bond hydrolyzed by trypsin, and to confirm that fragment A' is derived from fragment A, the N-terminal region of purified fragment A' was sequenced (see Experimental Procedures). The sequence Ser-Gly-Thr-Gln-Gly-Asn-Tyr-Asp-Asp-Asp- was obtained. By comparison to the published sequence of DT, the first residue must be Ser-40. Therefore, trypsin attacks after the lysine residue at the position 39. This gives a calculated molecular weight for fragment A' which is in good agreement with that determined by gel electrophoresis.

The effect of conversion of fragment A into A' upon biological activity was also examined. Figure 6 shows the $NAD^+$ glycohydrolase activity of free toxin is lost when there is complete cleavage at Lys-39 (i.e., upon conversion to an A'–B covalent complex). Isolated fragment A' also showed loss of the $NAD^+$ glycohydrolase activity present in isolated fragment A (data not shown). However, since fragments are isolated under denaturing conditions and then renatured, this latter observation could be due to a defect in fragment A' refolding. Ligand binding also appears to be either lost or greatly weakened upon cleavage at Lys-39. We found binding of the ligands ApUp and NADH to whole toxin was accompanied by considerable quenching (20–35%) of the Trp fluorescence of the toxin. This quenching was largely abolished after cleavage at Lys-39 (data not shown). These results indicate the activity-related functions of the A'–B complex are greatly decreased or lost. However, it should be noted that the loss of activity does not prove that a complex of the small peptide, which we can call peptide 1–39, with either fragment A' or the A'–B fragment would not function because we do not know if peptide 1–39 is present in our samples or whether it would bind sufficiently tightly to form a complex under our experimental conditions if present.

The studies above suggest that Lys-39 may be near the active site in DT. This is reinforced greatly by consideration of the homology between DT and PE. This homology is shown in Figure 7. It involves the N-terminal half of the A fragment of DT and the N-terminal half of the active domain of PE (see Figure 1). The amino acid identity is 27% in this region, allowing for introduction of three small gaps. Homology rises to 40% if the substitutions Val-Leu, Asp-Glu, and Lys-Arg are considered conservative changes, consistent with most homology scoring systems (Feng et al., 1985). In contrast, homology in the C-terminal halves of these domains is very weak unless many gaps are introduced, and introduction of these gaps results in misalignment of the functionally homologous region around Glu-148 in DT and Glu-553 in PE (Carroll & Collier, 1987). Several statistical analyses have been proposed in the literature to distinguish true homology from random similarities. Scoring homology by a modified log odds matrix or the structure–genetic method (Feng et al., 1985) indicates the observed level of homology between the N-terminal regions is indeed significant, giving scores of 905 and 372, respectively. Very high identity (≥40%) observed in stretches of at least 25 residues has also been suggested to be a definate indicator of significant homology (Dayhoff et al., 1983), and this relationship is found between A fragment residues 47–70 and the homologous stretches in PE. Given the functional similarity of PE and DT, it is reasonable to expect that the homology between them largely reflects active-site residues. Therefore, the fact that Lys-39 is in the well within the homologous sequence strongly suggests it is in or adjacent to the active site. This has in fact been at least partly confirmed by the identification of the apparent active-site cleft in the crystal structure of PE (Allured et al., 1986), which has been found to be the binding site for nicotinamide, and which is largely surrounded by the conserved residues (D. B. McKay and R. J. Collier, personal communication). In PE, Arg-458, the homologue of Lys-39 (see Figure 7), is a surface residue at the margin of the cleft, further supporting an active-site location for these residues. Interestingly, it is not adjacent to Glu-553, which has been identified as an active-site residue in contact with $NAD^+$ in PE (see the introduction). (The distance between Arg-458 and Glu-553 α-carbons is 18 Å.) However, $NAD^+$ is relatively large, extending roughly 15 Å when bound to dehydrogenases (Rossman et al., 1975), and steric blockage, or a very local change in cleft conformation upon $NAD^+$ binding, is still the likely cause of protection from trypsin attack at Lys-39.

## DISCUSSION

Combining the proteolysis results with the new information concerning homology between DT and PE allows us to make some conclusions about the structure of DT. First, the fact that it is a site of proteolysis suggests Lys-39 is at the surface of the DT molecule. This conclusion is strengthened by the fact that the crystal structure of PE shows that Arg-458, the residue homologous to Lys-39, is also at the surface of a domain. Second, the fact that both $NAD^+$ and ApUp block proteolysis at Lys-39 provides further evidence that they indeed

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      420                 440                 460                 480                 500
PE: TVERLLQAHRQLEERGYVFVGYHGTFLEAAQSIVFGGVRARSQ***DLDAIWRGFYIAGDPALAYGYAQDQEPDARGRIRNGALLRVYVP
DT: GADDVVDSSKSFV**MENFSSYHGTKPGYVDSIQKGIQKPKSGTQGNYDDDWKGFYSTDNKYDAAGYSVDNENPLSGK**AGGVVKVTYP
    1                 20                  40                  60                  80
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FIGURE 7: Sequence homology between diphtheria toxin and *Pseudomonas* exotoxin A. The first 86 residues from DT fragment A are shown aligned with sequences of the active fragment of PE. The residues underlined in both toxin sequences are identical. The residues underlined only in the DT sequence represent conservative changes. Gaps in alignment are represented by asterisks.

bind at overlapping or identical sites. Third, blockage of proteolysis by these ligands implies they either sterically block trypsin from approaching Lys-39 or cause a conformational change such that Lys-39 is considerably less exposed. The former interpretation is strongly supported by the homology analysis and crystal structure data described in detail above. In addition, it should be noted that although the loss of activity upon trypsin attack at Lys-39 cannot prove that Lys-39 is near the active site, because it may involve loss of a relatively large peptide (residues 1–39) from the A subunit, it does not contradict that conclusion.

As the above discussion indicates, the combined proteolysis, homology, and crystal structure data imply that the structures of the active site on DT and PE are likely to be similar. Furthermore, the apparent similarity between the location of DT Lys-39 in solution and that of Arg-458 in the crystal structure of PE indirectly implies a corresponding similarity between the crystal and solution structures of PE. This is of significance because in solution native PE must be artificially activated to express its enzymatic functions in vitro (Lory & Collier, 1980b; Collier et al., 1982), and therefore its crystal and enzymatically active structures could differ. It is also important to point out that the observations made in this report cannot precisely determine the location and any possible functional role of Lys-39. It should be emphasized that at present there is no evidence that Lys-39 has any role in catalysis, and it may not even directly contact $NAD^+$. We hope that the crystal structure of DT, now being solved in two laboratories (McKeever & Sarma, 1982; Collier et al., 1982), will give much more detail on the similarities between PE and DT active sites and the role of Lys-39. It would also be of interest to determine the location of elongation factor 2 binding relative to the apparent active site.

In addition to the homology between PE and DT, inspection of their sequences shows they share certain other peculiarities of amino acid composition. The most striking peculiarities involve His. As we noted previously, the A fragment contains only the 1 conserved His while the B fragment has 15 (London et al., 1986). As we speculated, this pattern may somehow reflect the catalytic function of DT, which attacks a modified His residue found on elongation factor 2 and ADP-ribosylates it on the His ring (Bodley et al., 1984). PE shows a similar pattern in its sequence with only 3 His residues in its C-terminal active fragment and none in its middle domains more C-terminal than the site of the disulfide bond between residues Cys-265 and -280, but with 12 His residues in its N-terminal half which, like the B fragment of DT, is believed to carry the receptor binding site (Allured et al., 1986; Hwang et al., 1987). In addition, in the conserved region, Lys residues are used in DT but not Arg residues, whereas PE uses only Arg both in this region and in the adjacent portion of its central domain which shows the His anomaly. Part of this difference may arise from the marked difference in the GC content of the organisms carrying each toxin, reflecting the fact that the PE gene is GC rich and Arg codons tend to use GC base pairs, whereas DT is AT rich, as are Lys codons. However, since the exclusive use of Lys or Arg is not found in the remainder of either of these proteins, additional factors could certainly be involved.

It should also be noted that the "loop" between Cys-265 and -280 of PE (Allured et al., 1986) has several adjacent basic residues (274–276) in its center, reminiscent of the cleavage site in the loop between A and B fragments of DT, and these residues form the boundary of the His-free, Lys-free domain (at their C-terminal side) and Lys and His using domains (on their N-terminal side). It would be interesting to know whether this boundary represents a functionally important cleavage site.

Finally, it is interesting to speculate whether the protective action of ApUp has any significance in vivo. It is conceivable that ApUp helps prevent an inactivating proteolytic attack on the A fragment, assuming ApUp is present in vivo. Certainly, binding of ApUp to toxin is extremely tight. On the other hand, at 37 °C (but not at 4 °C), dissociation of ApUp from toxin in vitro is significant, which suggest it could be lost in vivo (Collins et al., 1984). Nevertheless, since the physiological concentration of ApUp, if any, is unknown and the presence of additional factors could affect binding of ApUp under physiological conditions, whether or not ApUp remains associated with toxin in vivo and has a protective function cannot yet be decided.

## Added in Proof

It should be mentioned that DT Gly-52, which is within the homologous sequence, has also been suggested to be important in toxin interaction with $NAD^+$ (Giannini et al., 1984). Also, we have noticed that domain-specific bias toward Arg residues relative to Lys is found in a number of protein toxins in addition to PE, suggesting it may be of general importance (unpublished observations).

## Acknowledgments

We thank Royce Calhoun for technical assistance, Dr. Paul M. Bingham for helpful discussions, and Drs. D. B. McKay and R. J. Collier for providing us with the details of the crystal structure of PE. We also thank Dr. Collier for a copy of his manuscript with Dr. S. F. Carroll on the subject of DT/PE homology.

**Registry No.** Lys, 56-87-1; NAD, 53-84-9; ApUp, 1985-21-3.

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