Histidine 21 Does Not Play a Major Role in Diphtheria Toxin Catalysis*

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It has been proposed that the histidine at position 21 (H21) of the diphtheria toxin A subunit (DTA) plays an important role in the ADP-ribosyltransferase (ADPRT) activity of the toxin. The region of DT encompassing H21 demonstrates sequence similarity with other toxins exhibiting ADPRT activity, is located along the catalytic cleft of DTA, and when H21 is chemically modified, ADPRT activity is abolished. H21 was mutagenized by a polymerase chain reaction-based system whereby all alternative amino acids were substituted in place of the histidine. The majority of the substitutions virtually abolished enzymatic activity, the exception being a mutant in which H21 was replaced with asparagine (DTA-H21N). This mutant demonstrated only a slight increase in $k_{cat}$ and relatively small decreases in both reaction rate ($k_{cat}$) and catalytic efficiency ($k_{cat}/K_m$). Asparagine is a sterically conserved substitution, but its side-chain is unable to replace the imidazole group of histidine in general acid-base mechanisms or to participate in electrostatic interactions. This suggests that H21 is important in maintaining a steric conformation required for catalysis rather than in participating in an electrostatic or acid-base type of exchange.

Diphtheria toxin (DT) is a family of bacterial toxins known as ADP-ribosyltransferases (ADPRT). It catalyzes the transfer of ADP-ribose to a post-translationally modified histidine residue, diaphamidine, found in elongation factor 2 (EF-2) (Honjo et al., 1968). The ADP-ribosylated EF-2 can no longer mediate polypeptide chain elongation and as a result protein synthesis ceases and the intoxicated cell ultimately dies.

Despite the fact that DC was the first of the ADP-ribosylating toxins for which the enzymatic activity was identified (Honjo et al., 1968), relatively little is known about the catalytic mechanism or the active site of the toxin. DT contains a single high affinity NAD+ binding site ($K_f = 8 \mu M$) (Kandel et al., 1974). Binding of NAD+ is rapidly reversible and no evidence has been found for a covalent ADPR-DTA intermediate (Kandel et al., 1974). Some sites proposed to be important for catalytic activity (e.g. G52, G128) were identified using nitrosoquinidine mutagenesis (Uchida et al., 1973). Another postulated critical site (E148) was identified by photoaffinity labeling (Carroll and Collier, 1984) and was later confirmed by replacement (Twitchen et al., 1985; Barbieri and Collier, 1987; Wilson et al., 1990; O'Keefe, 1992) and deletion mutagenesis (Emerick et al., 1995; Killeen et al., 1992).

H21 has also been proposed to be a critical residue for the enzymatic activity of DT. This is based on a number of observations. 1) Analysis of the crystal structure of DT reveals that H21 is located within what is believed to be the enzymatic cleft (Choe et al., 1992). 2) The area around H21 has sequence similarity with other toxins that act as ADPRT, including Pseudomonas exotoxin A, pertussis toxin S1 subunit, cholera toxin, and Escherichia coli heat labile toxin (Gill, 1986; Carroll and Collier, 1988; Domenighini et al., 1991). 3) H21 in DT has been chemically modified with 4-dimethyl pyrocarbonate, resulting in an inhibition of toxicity (Papini et al., 1989). In order to better understand the structure-function relationships of DT and the potential role of H21 in the enzymatic activity of DT, we initiated a series of studies aimed at the mutagenesis of this residue. All alternative amino acids were substituted for H21 to give a thorough understanding of the role that H21 plays in DT catalysis.

EXPERIMENTAL PROCEDURES

Reagents—DT was purchased from List Biologicals (Campbell, CA). The TNT rabbit reticulocyte lysate-based coupled transcription/translation system, recombinant RNAasin, and plasmid purification columns were obtained from Promega (Madison, WI). Reagents for PCR were from Perkin-Elmer. Tissue culture supplies and restriction enzymes were purchased from Life Technologies Inc. [32P]ATP was from DuPont-New England Nuclear. [adenyl-32P]NAD+ was supplied by ICN Biomedicals (Costa Mesa, CA). Corynebacterium diphtheriae strains C7β (ATCC 27012) and C7β(197) (ATCC 53281) were obtained from the American Type Culture Collection (Rockville, MD). The cloning vector Bluescript KS+ and the host cell line XLI-Blue were from Stratagene (La Jolla, CA). PEF-11d and its expression host, B21DE3, were purchased from Novagen (Madison, WI).

Construction of Plasmid Libraries Randomly Mutated at Residue 21—A diagrammatic representation of the cloning procedure used to generate the library of mutants is shown in Fig. 1. The number of amino acid residues is as defined by Greenfield et al. (1983). Genomic DNA was obtained from C. diphtheriae C7β (Roth) as previously described (Johnson et al., 1983), and the region of the tox gene encoding DTA was amplified in two halves, using two pairs of PCR primers; the first pair (1 and 2a) were designed to amplify the 5′ portion of the gene (nucleotides encoding residues 1–30). Primer 1 included a BamHI site for cloning, in addition to a translation initiation codon in the optimum Kozak context immediately prior to the first nucleotide of the DTA gene. Random substitutions were generated at nucleotides 61–63 (encoding amino acid residue 21) using primer 2a, as shown in Fig. 1. The 3′ portion of the DTA gene (nucleotides encoding residues 122–193) was amplified using the second pair of primers (2b and 3) which made no nucleotide substitutions, but generated a product which had 27 nucleotide homology at its 5′ end with the 3′ end of the primer 1–2a product.
(the stippled region in Fig. 1). Primer 3 was designed to include an EcoRI site for cloning, and to introduce tandem translation stop codons immediately after the third nucleotide of codon 193. The two halves of the gene were then spliced together using a PCR-based method as previously described (Johnson et al., 1993; Nicholls et al., 1993). Each round of PCR was comprised of 20 cycles. The corresponding region of the gene encoding CRM 197, a mutant of DT with no enzymatic activity, was also amplified using primers 1 and 3. Products were digested with BamHI and EcoRI, and treated with alkaline phosphatase, prior to cloning into the Bluescript KS+ vector in a region downstream of the T7 RNA polymerase promoter. The inserted region encodes for amino acids 1–193 and corresponds to the enzymatically active form of DTA (Williams et al., 1990). Genes encoding DTA were cloned without DTB in compliance with the restrictions on cloning toxin genes (Federal Register, 1990). E. coli strain XL1-Blue was transformed with the recombinant library. Plasmid DNA from the resulting colonies was isolated and used directly as a template for transcription/translation, and for sequencing.

In Vitro Transcription/Translation of DNA Templates—Plasmid templates were transcribed and translated using a rabbit reticulocyte lysate-based coupled transcription/translation system, according to the instructions of the manufacturer. [35S]<sup></sup>Metionine-labeled proteins were quantitated by trichloroacetic acid precipitation and analyzed by SDS-PAGE. Radiolabeled proteins were also used for the determination of enzymatic activity.

ADP-ribosyltransferase Assay—[<sup>35S</sup>Met]Metionine-labeled proteins were quantitated by trichloroacetic acid precipitation and the enzymatic activities of the various DTA mutants were determined as previously described (Johnson et al., 1988). The counts obtained from the trichloroacetic acid precipitation of H21M were adjusted to take into consideration the fact that this mutant has an extra methionine residue. [35S]<sup></sup>Met-labeled proteins could be used in the ADPR assay because counting <sup>35</sup>P-labeled EF-2 by the Cerenkov method does not register the weaker emissions of <sup>35</sup>S (data not shown).

Expression of DTA Mutants in E. coli—DTA mutants were expressed in E. coli BL21(DE3), using the T7 RNA polymerase-driven expression vector, pET-11d (Studier et al., 1990). Genes encoding mutants of interest from the original library were modified by PCR to include a region encoding 6 histidine residues ([His]6) immediately following codon 197. The two halves of the gene resulting from the first round of PCR are spliced together in the second or recombinant round. Primers 1 and 3 include a BamHI and EcoRI site, respectively, for use in cloning the PCR products. Each round of PCR was comprised of 20 cycles.

carried out at 37 °C for a total of 3 min, precipitated with 10% trichloroacetic acid and Cerenkov counted. The kinetic parameters were obtained by analysis of Lineweaver-Burk plots from at least three separate experiments, each performed in duplicate.

SDS-PAGE—SDS-PAGE was carried out using 10–20% gels (Daichi, Tokyo, Japan) as described by Laemmli (1970). For analysis by fluorography, the gels were fixed for 20 min in 10% methanol, 7% acetic acid, treated with Amplify (Amersham) for 30 min, and dried. Gels were exposed to X-Omat AR2 film (Eastman Kodak, Rochester, NY) in the absence of an intensifying screen.

Other Methods—Trypsin digestion patterns of wild-type or mutant DTA were compared by premixing 4 μg of toxin with 200 μm NAD<sup></sup><sup></sup>+, digested with varying concentrations of trypsin (Boehringer Mannheim) for 1 h at 37 °C, and analyzing by SDS-PAGE.

Dideoxynucleotide chain-termination sequencing of double-stranded DNA templates was performed using a Sequenase II kit (U. S. Biochemical Corp.), according to the manufacturer’s protocol.

RESULTS

Mutagenesis of H21—H21 was mutagenized using a PCR-based method (Fig. 1). By introducing totally random nucleotide substitutions at positions 61 through 63, it was possible to generate a library of mutants encoding all possible amino acids at codon 21. PCR products were cloned into the Bluescript KS+ vector in a region downstream of the T7 RNA polymerase promoter. The presence of this promoter enabled initial toxin production using an in vitro coupled transcription/translation system, facilitating screening of a large number of DTA clones.

Equal volumes of radiolabeled products from the coupled transcription/translation system were analyzed by SDS-PAGE (Fig. 2). Under reducing conditions, wild-type DTA as well as the mutant forms of DTA produced a major band with molecular weight of about 21,000. In addition, a lower band of consis-

![Fig. 1. PCR-based mutagenesis. The tox gene from wild-type C. diphtheriae genomic DNA corresponding to nucleotides coding for residues 1–193 was amplified in two halves. Totally random nucleotide substitutions (NNN, corresponding to nucleotides 60–63) were used in primer 2a thus enabling the replacement of H21 encoded by (CA)<sup>2</sup> with other amino acids. The two halves of the gene resulting from the first round of PCR are spliced together in the second or recombinant round. Primers 1 and 3 include a BamHI and EcoRI site, respectively, for use in cloning the PCR products. Each round of PCR was comprised of 20 cycles.](image-url)
band results from translation initiation at codon 14 was obtained by modifying primer 1 (Fig. 1) so that it deleted the first 13 codons of the DTA chain and instead started at the ATG coding for residue M14. The protein produced ran as a single band after SDS-PAGE, migrating to precisely the same location as the lower M band (data not shown).

Clones producing a translation product of the correct size were sequenced across the region spanning the mutagenized codon to determine the identity of the substituted amino acid. Clones containing all 20 possible amino acids at position 21 were isolated. The mutant genes were subjected to a total of 40 rounds of PCR, therefore, the potential for PCR-induced errors in regions that were not sequenced exists. However, only five unexpected mutagenic events were observed in approximately 13,000 bases sequenced; the probability of there being a single error in a given DTA clone is therefore approximately 1 in 4. Although there is a possibility that a PCR error in a site other than H21 could affect enzymatic activity, we have isolated multiple independent clones for the majority of the amino acid substitutions, and all exhibited identical activity for a given substitution.

Analysis of Enzymatic Activity—Radiolabeled translation products for wild-type DTA, CRM 197, and the DTA mutants were quantitated by trichloroacetic acid precipitation and comparable amounts of protein compared using a cell-free ADP-ribosylation assay (Fig. 3). A clone containing histidine at position 21 (DTA-H21H) was isolated and found to have comparable enzymatic activity to wild-type DTA generated by subjecting genomic DNA to 20 cycles of PCR amplification using primers 1 and 3 (Fig. 1), cloning the PCR product into Bluescript KS, and expressing in vitro. CRM 197, a mutant containing a single mutation at amino acid position 52 (G52E) resulting in the inability to bind NAD+ and a total lack of enzymatic activity, was also included as a negative control.

Multiple clones were isolated for the majority of the amino acid substitutions (all except V, Q, and E). DTA mutants were analyzed in duplicate in three separate ADP-ribosylation assays and the results were plotted as the percent of wild-type enzymatic activity (Fig. 3). The majority of the amino acid substitutions resulted in mutants with extremely low levels of ADPRT activity; substitution with A, V, L, I, P, F, W, M, G, Y, Q, D, E, or K resulted in mutants expressing less than 5% of wild-type ADPRT activity under the conditions of the assay. Substitution of S, T, C, or R at position 21 resulted in enzymatic activity between 5 and 10% of wild-type. The one exception with significant activity was DTA-H21N which demonstrated 65% of the wild-type enzymatic level.

The data obtained from mutants produced in vitro was a valuable guide in the selection of candidates for further characterization. However, a more accurate determination of the kinetic parameters of the ADPRT reaction required larger quantities of pure protein at known concentration. Since the yields obtained from in vitro expression systems are limited, selected mutants were expressed in E. coli to produce sufficient quantities of toxin to allow a more detailed characterization.

Expression of Selected H21 Mutants in E. coli—H21 mutants to be expressed in E. coli were selected on the basis of their relative enzymatic activity. DTA-H21N was chosen because it was the only mutant isolated with significant enzymatic activity. DTA-H21A was selected because it demonstrated low but measurable enzymatic activity (about 5% wild-type). DTA-H21F and DTA-H21G both displayed undetectable enzymatic activity in a cell-free assay at the concentration generated in vitro. The phenylalanine and glycine mutants have very different sized side-chains at position 21, and may have significantly different effects on main-chain conformation, despite both essentially abolishing enzymatic activity. The effect of amino acid substitutions on the overall conformation of the toxin was an important consideration in this study; DTA mutants that are enzymatically inactive but retain the wild-type conformation could prove to be useful as potential vaccine candidates.

These four mutants together with wild-type (DTA-H21H) and CRM 197 DTA were selected for expression in E. coli BL21(DE3) using the pET-11d vector. The selected genes were modified by PCR to include a region encoding 6 histidine residues immediately following codon 197 to facilitate purification (Porath, 1992). All constructs were completely sequenced to verify the amino acid substitution and to confirm the remainder of the DTA chain sequence. The corresponding DTA proteins were produced in E. coli and purified to >95% homogeneity using immobilized metal chelate affinity chromatography (Porath, 1992) (Fig. 4).

To test whether the presence of the (His)6 tail has any influence on enzymatic activity or conformation, toxins were digested with immobilized carboxypeptidase A to remove residues from the COOH terminus of the proteins. Cleavage terminated at Arg-193, the new basic residue encountered by the enzyme. Complete removal of the histidine tail was confirmed by the inability of carboxypeptidase A-digested proteins to bind to the metal chelating resin. The presence of the histidine tail did not alter the enzymatic or conformational proper-
ties of the toxins (data not shown).

Trypsin sensitivity of DTA-H21H was compared to that of the four mutants. The amino acid changes at position 21 caused no significant increase in sensitivity to trypsin implying that these substitutions produced little or no alteration in the overall protein structure (data not shown).

Enzymatic Analysis of Selected DTA-H21 Mutants—A comparison of the ADPRT activities of selected H21 mutants at varying toxin concentrations is shown in Fig. 5. Activities relative to wild-type were based on the concentration of enzyme required to ADP-ribosylate 50% of the available EF-2, under the conditions of the assay. The activity of DTA-H21H produced in *E. coli* was comparable to that of commercially purchased DT (data not shown). DTA-H21N was approximately 30-fold less enzymatically active than native DTA-H21H, and DTA-H21A was approximately 230-fold less active. Full ADPRT activity curves could not be generated for DTA-H21F or DTA-H21G due to limitations in toxin concentration. However, estimation of their relative enzymatic activities by extrapolation suggests that DTA-H21F was approximately 1500-fold and DTA-H21G was approximately 4000-fold less enzymatically active than DTA-H21H. Supporting preliminary data generated by *in vitro* expression of these mutant A chains with wild-type DTB suggests that toxicity on a DT-sensitive cell line follows the same pattern as enzymatic activity. (Fig. 5A).

A detailed analysis of the kinetic aspects of enzymatic activity was also carried out. *K*ₘ values for wild-type and wild-type toxins in the ADPRT reaction were determined from initial rate data obtained at fixed EF-2 concentrations. The kinetic parameters obtained by analysis of Lineweaver-Burk plots of initial reaction velocities are summarized in Table I. The *K*ₘ for wild-type DTA-H21H was 9 μM, corresponding to the value previously published for DT (Kandel et al., 1974). DTA-H21N and DTA-H21F both show a slight increase in *K*ₘ (17 and 25 μM, respectively). In contrast, *K*ₘ for DTA-H21A and DTA-H21G was greatly increased (63 and 48 μM, respectively), suggesting that these mutants had significantly reduced affinity for NAD⁺.

The kinetic parameters *k*ₐ and *k*ₐ/ *K*ₘ were also calculated for the wild-type toxin and the mutants (Table I). The asparagine substitution at position 21 resulted in an enzyme with only 67- and 457- and 913-fold reduction, respectively, in catalytic rate (67-, 457-, and 913-fold reduction, respectively, in wild-type poses a serious safety problem (Killeen et al., 1992), and it is therefore important to have a thorough understanding of the effect of all possible amino acid substitutions at the site of mutagenesis. The technique circumvents problems encountered in selecting the amino acid to replace the native residue and gives a more complete view concerning the role that a particular residue plays. Mutagenesis of DT could potentially produce mutants with impaired enzymatic activity yet with the native conformation, and such mutants could prove to be useful vaccine candidates. In a vaccine context, it is becoming increasingly clear that the potential for reversion back to wild-type poses a serious safety problem (Killeen et al., 1992), and it is therefore important to have a thorough understanding of the effect of all possible amino acid substitutions at the site of mutagenesis. The PCR-based method, together with *in vitro* expression of the resulting mutants and analysis by a cell-free ADPRT assay, allows an easy and rapid method of screening a large number of mutants. It also provides essential data required for safety assessment prior to large scale in vivo culture of toxins.

H21 was selected for mutagenesis, because this residue has been proposed to play an important role in ADPRT activity of DT. Of the 16 histidine residues present in DT, it is the only one found within the A chain of the toxin. Analysis of the crystal structure of DT reveals that H21 is located within the enzymatic cleft of DT (Choe et al., 1992). The histidine imidazole ring is believed to project to the inside of this cavity, thus facilitating its involvement in hydrogen bonding (Domenighini et al., 1991). The region encompassing H21 also demonstrates sequence similarity with other toxins expressing ADPRT activity, including *Pseudomonas* exotoxin A, pertussis toxin S1 subunit, cholera toxin, and *E. coli* heat labile toxin (Gill, 1988; Carroll and Collier, 1988; Domenighini et al., 1991). The corresponding residue of the S1 subunit of pertussis toxin (H35) has been mutated resulting in a marked reduction in ADPRT activity.
activity (Kaslow et al., 1989).

Treatment of DTA with diethyl pyrocarbonate selectively modifies H21 and results in inhibition of NAD+ binding and ADPRT activity (Papini et al., 1989), suggesting that H21 is involved in the enzymatic activity of DT. Nevertheless, without site-directed mutagenesis studies directed at this residue, the possibility that these results are due to steric hindrance from the bulky chemical modification cannot be discounted (Miles et al., 1989).

In an attempt to clarify the role of H21 in catalysis, all alternative amino acid substitutions were substituted in place of H21 and the results of these mutations on ADPRT activity were analyzed. DTA-H21N was the only mutant that retained significant enzymatic activity. When DTA-H21N was assayed at varying toxin concentrations, it was found to be only 30-fold less enzymatically active than wild-type DTA. Similarly, its K_m was increased only slightly, thereby indicating that its ability to bind NAD+ is not significantly impaired. The catalytic rate (k_cat) and efficiency (k_cat/K_m) are also only reduced slightly relative to wild-type toxin. This is interesting considering that H21 also has been proposed to function in ADPRT activity through its interaction with E148 (Wilson et al., 1990; Wilson and Collier, 1992). A titratable group with a pK_a of 6.2–6.3 (Papini et al., 1990) was identified within DTA and assumed to represent the protonation of the histidine. It was proposed that the carboxyl group of E148 strongly affects this titration, and maintains a particular active-site conformation possibly by confining the imidazole ring of H21 in the position required for catalysis. Alternatively, E148 may alter the nucleophilicity of the incoming diphthamide through acid-base interaction with the histidine. Asparagine is assumed to be a sterically conservative substitution for histidine, but the sidechain of asparagine can neither replace the imidazole group of histidine in general acid/base mechanisms nor participate in electrostatic interactions. The fact that asparagine can substitute for histidine at position 21 suggests that histidine is important in maintaining the required conformation rather than in participating in an electrostatic or acid-base type of exchange.

The majority of amino acids substituted at position 21 virtually abolish ADPRT activity. DTA-H21A and DTA-H21G both show significant increases in K_m as well as decreases in both the catalytic rate (k_cat) and efficiency (k_cat/K_m) of ADPRT. It is noteworthy that DTA-H21A has the highest K_m value, although it is by no means the least efficient enzyme (the phenylalanine and glycine mutants are approximately 3- and 11-fold less efficient than DTA-H21A, respectively). Alanine does not have a side-chain beyond the -beta-carbon and therefore is not believed to alter the main-chain conformation or impose extreme electrostatic or steric effects (Ashkena et al., 1990). Nonetheless, the H21A substitution results in an inability to maintain the ideal conformation required for catalytic activity. For DTA-H21F, K_m was only slightly higher than for the asparagine mutant, yet catalytic efficiency was significantly lower than for DTA-H21N. This suggests that the bulky side-chain of phenylalanine does not cause a drastic decrease in ability to bind NAD+, but that the conformation of the bound substrate is not ideal with respect to the catalytic mechanism. These conformational changes appear to be subtle since the amino acid substitutions analyzed do not appear to result in gross structural changes as demonstrated by sensitivity to trypsin proteolysis.

It has recently been shown that the deletion of an active site residue drastically reduces ADPRT activity, although activity can be partially restored by second-site mutations (Killeen et al., 1992). Clearly, in the context of a potential vaccine candidate, reversion of an inactive mutant to an enzymatically active form would pose a serious safety problem. Therefore, multiple mutations, each independently detoxifying the molecule, may be required for the construction of a genetically inactivated toxin for use as a vaccine. A substitution at position 21 may prove useful as one of these mutations. H21G has greatly reduced enzymatic activity (rel. k_cat/K_m is reduced about 5000-fold), yet based on trypsin sensitivity, is believed to have a conformation similar to that of the wild-type toxin. Work is in progress to further analyze this mutant and others as potential vaccine candidates.

Acknowledgments—We thank Drs. William Habig and Jerry Keith for continued support and useful discussions. We thank Sheila Halsey for the gift of E-2 and acknowledge Drs. Mike Brennan, Jacob Donker-sloot, Claudia Gentry-Weeks, and Jane Halpern for constructive comments on the manuscript.

Addendum—Another group has independently reported that asparagine substitution for H21 has >25% enzymatic activity (Blanke et al., 1992).

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| TABLE I |
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| Kinetics of ADP-ribosyltransferase activity |
| | DTA-H21H | DTA-H21N | DTA-H21A | DTA-H21F | DTA-H21G |
| K_m (μM) | 9 | 17 | 63 | 25 | 48 |
| V_max (umol/min/mg) | 1.55 | 9.4 x 10^-2 | 2.4 x 10^-2 | 3.4 x 10^-3 | 1.7 x 10^-3 |
| k_cat (min^-1) | 34.7 | 2.11 | 0.537 | 0.076 | 0.038 |
| rel. k_cat | 1.0 | 6.1 x 10^-2 | 1.5 x 10^-2 | 2.2 x 10^-4 | 1.1 x 10^-4 |
| k_cat/K_m (s^-1/mn^-1) | 3.85 x 10^6 | 1.22 x 10^6 | 8.52 x 10^5 | 3.04 x 10^6 | 7.92 x 10^6 |
| rel. k_cat/K_m | 1.0 | 3.2 x 10^-2 | 2.2 x 10^-3 | (1/452) | (1/1266) | (1/4861) |
