Engineering Functional Changes in *Escherichia coli* Endonuclease III Based on Phylogenetic and Structural Analyses*

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*Escherichia coli* endonuclease III (EcoNth) plays an important cellular role by removing premutagenic pyrimidine damages produced by reactive oxygen species. EcoNth is a bifunctional enzyme that has DNA glycosylase and apurinic/apyrimidinic lyase activities. Using a phylogeny of natural sequences, we selected to study EcoNth serine 39, aspartate 44, and arginine 184, which are presumed to be in the vicinity of the damaged base in the glycosylase-substrate complex. These three amino acids are highly conserved among Nth orthologs, although not among homologous glycosylases, such as MutY, that have different base specificities and no lyase activity. To examine the role of these amino acids in catalysis, we constructed three mutants of EcoNth, in which Ser39 was replaced with leucine (S39L), Asp44 was replaced with valine (D44V), and Arg184 was replaced with alanine (R184A), which are the corresponding residues in EcoMutY. We showed that EcoNth S39L does not have significant glycosylase activity for oxidized pyrimidines, although it maintained AP lyase activity. In contrast, EcoNth D44V retained glycosylase activity against oxidized pyrimidines, but the apparent rate constant for the lyase activity of EcoNth D44V was significantly lower than that of EcoNth, indicating that Asp44 in EcoNth is required for β-elimination. Finally, EcoNth R184A maintained lyase activity but exhibited glycosylase specificity different from that of EcoNth. The functional consequences of each of these three substitutions can be rationalized in the context of high resolution protein structures. Thus phylogeny-based scanning mutagenesis has allowed us to identify novel roles for amino acids in the substrate binding pocket of EcoNth in base recognition and/or catalysis.

Base excision repair evolved to protect cellular DNA against the deleterious effects of endogeneous metabolic processes and is highly conserved across species (for reviews, see Refs. 1–3). The initial step in the base excision repair pathway is the removal of a damaged pyrimidine or purine by cleavage of the N2-glycosyl bond by a DNA glycosylase. The glycosylases that recognize oxidative damages also exhibit AP2 (apurinic/apyrimidinic) lyase activity, the ability to cleave the DNA backbone. *Escherichia coli* endonuclease III (EcoNth) can excise a variety of damaged pyrimidines and formamidopyrimidines and is a bifunctional enzyme that contains both glycosylase and AP lyase activities (1–3). Homologs of Nth are present in all three phylogenetic domains. Two archaeal enzymes have been purified and characterized biochemically. Nth from the hyperthermophilic archaeon, *Pyrobaculum aerophilum* (PaNth), exhibits glycosylase/β-lyase activity on dihydrothymine (4), while Nth from * Archaeoglobus fulgidus* (AfNth) has been examined by nuclear magnetic resonance for its interaction surfaces with a reduced AP site-containing DNA substrate (5). Eukaryotic orthologs have been studied in a number of organisms. In the yeast *Saccharomyces cerevisiae*, there are two Nth orthologs, Ntg1 and Ntg2, and like EcoNth, both enzymes are bifunctional and cleave a wide variety of oxidized substrates (6–9). An Nth ortholog has been cloned from *Schizosaccharomyces pombe*, Nth-Spo (10, 11). The bovine, mouse, and human orthologs of EcoNth have also been cloned and the proteins characterized (12–16). Like EcoNth, bNTH1, bNth, and mNth1 possess DNA-glycosylase/lyase activity on oxidized pyrimidines, formamidopyrimidines, and AP sites. Thus Nth is conserved over all three phylogenetic domains, and with respect to qualitative functional features (that is, glycosylase and lyase activities), EcoNth represents the activity of orthologous proteins and their common ancestor.

A closely related family member, EcoMutY, is a mismatch repair DNA glycosylase that removes adenine misincorporated opposite G, C, or 7,8-dihydro-8-oxoguanine during replication (17, 18). The major function of EcoMutY is to prevent mutation fixation when 7,8-dihydrox-8-oxoguanine mismatches with A, thus avoiding potential G → T transversions (19). Unlike EcoNth, however, EcoMutY is a monofunctional DNA glycosylase (20). The genes for the human (21, 22) and murine (23) homologs of EcoMutY have been cloned, and their substrate specificities are the same as that of EcoMutY. Interestingly, although EcoNth and EcoMutY share no common substrates, they have significant sequence similarity at the amino acid level (Fig. 1A) (24). When the crystal structure of the archetypical member of the Nth family, EcoNth, was solved (25, 26), a number of motifs were identified that are shared by relatives of EcoNth. The helix-hairpin-helix (HhH) motif, followed by a Gly/Pro-rich loop and a conserved aspartic acid (HhH-GPD motif), and the iron sulfur cluster loop, are conserved in almost all of the Nth orthologs (26). The iron sulfur cluster does not undergo any redox reactions but appears to be involved in DNA binding (26). EcoMutY also contains both the HhH motif and the iron sulfur cluster (24, 27). Thus, it appears that Nth and MutY were derived from a common ancestral gene.

Several amino acids have been implicated in the reaction chemistry of these proteins. For example, in EcoNth, Lys120 and Asp139, and in EcoMutY, Asp138, have been shown to be involved in catalysis (25, 26, 28). It has recently been proposed that Asp44 in *Bacillus steatorrhophilus*, homologous to Asp44 in EcoNth is also involved in catalysis (29). However, neither the role of particular amino acids in substrate binding nor the evolution of the DNA glycosylases is understood in detail. Based on
substitutions observed during natural sequence evolution (TABLE ONE) we have chosen site-directed EcoNth mutants to study the sequence basis of glycosylase specificity and lyase activity. Here we examine three such substitutions, EcoNth S39L, D44V, or R184A, that are in the vicinity of the substrate binding site (Fig. 1).

We show that EcoNth D44V retained glycosylase activity but had greatly reduced AP lyase activity relative to EcoNth. EcoNth S39L retained lyase activity but lacked glycosylase activity. Finally EcoNth R184A exhibited a qualitatively different substrate specificity from that of EcoNth.

**MATERIALS AND METHODS**

**Phylogeny-based Scanning Mutagenesis**—Proteins containing domains homologous to EcoNth were identified using BLAST family tools (30) and filtered manually. Multiple sequence alignments were constructed by manual modification of ClustalW (31) alignments. Phylogenies were constructed using PHYLIP (32) protml and neighbor. Occurrence of mutations associated with each aligned sequence position and edge was inferred by maximum parsimony using MacClade. One functional change was assumed to occur along the edge separating the Nth and MutY clades. Positions were identified at which substitutions were correlated with the functional change. A detailed description of phylogeny-based scanning mutagenesis is in preparation.

**Generation of the EcoNth Variants**—Site-directed mutagenesis was performed using the PCR. A part of the nth gene on plasmid pET22b was amplified using primers purchased from Qiagen, which contained the site of the desired mutation. A fragment containing one part of the nth gene that had Ser39 substituted with Leu or Asp44 substituted with Val was ligated to a vector containing the other part of the nth gene with two PshA1 (New England Biolabs Inc.) sites. In the same manner, a PCR product containing one part of the nth gene that had Arg184 substituted with Ala was ligated to a vector containing the other part of the nth gene with two BspMI (New England Biolabs Inc.) sites. The direction of insertion was checked with BglII restriction enzyme (New England Biolabs Inc.). The DNA sequences of EcoNth S39L, D44V, and R184A were confirmed using an ABI 373 sequencer.

**Enzymes**—EcoNth and three variants (S39L, D44V, or R184A) were overexpressed in E. coli strain lacking Fpg proteins and harboring plasmid pET22b containing either the nth, nth s39l, nth d44v, or r184a genes tagged on the C terminus with hexahistidine. The E. coli strain harboring each plasmid was incubated for 20 h at 16 °C with 0.3
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FIGURE 2. A, glycosylase/AP lyase activities of EcoNth and EcoNth S39L, D44V, and R184A. Enzymes (100 nM) were incubated with 5′-32P-labeled duplex oligonucleotides (10 nM) containing Tg:A (lanes 1–5), S-OHC:G (lanes 6–10), S-OHU:G (lanes 11–15), DHU:G (lanes 16–20), or DHT:A (lanes 21–25) at 37 °C for 10 min. Lanes 1, 6, 11, 16, and 21, no enzyme; lanes 2, 7, 12, 17, and 22, EcoNth; lanes 3, 8, 13, 18, and 23, S39L; lanes 4, 9, 14, 19, and 24, D44V; lanes 5, 10, 15, 20, and 25, R184A. Formamide dye was added to all samples after reaction. B, glycosylase activity of EcoNth and EcoNth S39L, D44V, and R184A. Enzymes (100 nM) were incubated with 5′-32P-labeled duplex oligonucleotides (10 nM) containing Tg:A (lanes 1–5), S-OHC:G (lanes 6–10), S-OHU:G (lanes 11–15), DHU:G (lanes 16–20), or DHT:A (lanes 21–25) at 37 °C for 10 min. Lanes 1, 6, 11, 16, and 21; no enzyme; lanes 2, 7, 12, 17, and 22, EcoNth; lanes 3, 8, 13, 18, and 23, S39L; lanes 4, 9, 14, 19, and 24, D44V; lanes 5, 10, 15, 20, and 25, R184A. NaOH was added to all samples after the reaction was completed. Samples were then treated at 95 °C for 5 min followed by addition of formamide dye.
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Enzyme Activity of Wild Type EcoNth and EcoNth R184A for Tg:A, Tg:G, DHU:A, or DHU:G—Wild type EcoNth or EcoNth R184A was incubated with substrates containing Tg:A, Tg:G, DHU:A, and DHU:G and the products analyzed by denaturing PAGE. The amount of nicked product produced was plotted as a function of enzyme concentration. Fig. 4A shows that under multiple turnover conditions and at low concentrations of enzyme, wild type EcoNth efficiently cleaved Tg:A, Tg:G, and DHU:G, but DHU:A was a much poorer substrate (see also Table TWO). Fig. 4B shows that for EcoNth R184A, under conditions of otheriol, and 10% glycerol at 37 °C for 15 min. 5 μl of the sample was loaded onto a 6% nondenaturing polyacrylamide gel containing 0.25 × TBE (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM EDTA, pH 8.0) and run at 300 volts for 90 min at 4 °C. The shifted 32P-labeled complexes and cleaved substrates were visualized by autoradiography.

NaCNBH₃ Trapping of Schiff Base Intermediates—EcoNth, EcoNth S39L, EcoNth D44V, or EcoNth R184A was incubated with substrates containing a DHU opposite G or an AP site opposite G in Reaction Buffer minus NaCl. Final enzyme concentrations were 50 nM. Reactions were performed in the presence of 50 mM NaCNBH₃ at 37 °C for 30 min. Samples were then mixed with SDS-PAGE loading buffer (125 mM Tris-HCl (pH 7.6), 10% 2-mercaptoethanol, 4% SDS, 10% glycerol, and 0.004% bromphenol blue) and heated to 90 °C for 5 min. The products were separated by 10% SDS-PAGE.

RESULTS

Bifunctional Glycosylase/Lyase Activity—When DNA containing an oxidized pyrimidine (Tg, 5-OHC, 5-OHU, DHU, or DHT) was incubated with wild type EcoNth (Fig. 2A), significant cleavage of each of the substrates was detected. On the other hand, Fig. 2A shows that in the absence of NaOH no significant cleavage by EcoNth S39L or EcoNth D44V was observed for any substrate tested. Denaturing gel analysis of the cleaved products produced by EcoNth R184A on a substrate containing DHU opposite G showed that the glycosylase/lyase activity (Fig. 2A, lane 20) was similar to that of EcoNth (lane 17). However, the activity of EcoNth R184A for Tg, 5-OHC, 5-OHU, and DHT (lanes 5, 10, 15, and 25) was much lower compared with that of EcoNth (lanes 2, 7, 12, and 22). Like wild type EcoNth, R184A yielded a β-elimination product.

Monofunctional Glycosylase Activity—The absence of substrate cleavage by EcoNth S39L or D44V is consistent with loss of glycosylase activity, loss of lyase activity, or loss of both activities. To directly determine glycosylase activity, S39L and D44V reactions were treated with sodium hydroxide to cleave any abasic sites. Fig. 2B shows the reactions of substrates containing an oxidized pyrimidine (Tg, 5-OHC, 5-OHU, DHU, and DHT) incubated with either wild type EcoNth or the three EcoNth variants followed by treatment with NaOH. The amount of β-elimination product for EcoNth without or with NaOH was similar (Fig. 2, A and B) as expected for a bifunctional glycosylase/lyase. EcoNth S39L did not cleave substrate even after addition of NaOH to the reacted samples (Fig. 2B). Thus it appears that S39L lost glycosylase activity on the oxidized pyrimidine substrates tested although substrates containing an AP site opposite A or G were cleaved by S39L (Fig. 3A). In contrast when NaOH was added to the reaction product after incubation with EcoNth D44V, cleavage was readily detected (Fig. 2B). Thus D44V retained monofunctional glycosylase activity producing an AP site after removing the oxidized pyrimidine. In keeping with this observation, when DNA containing an AP site opposite G or A was incubated with D44V (Fig. 3A), cleavage was substantially reduced compared with wild type EcoNth (compare lanes 2 and 4 with lanes 7 and 9). The same results were obtained when a time course was measured for wild type EcoNth and D44V (Fig. 3B), which showed about a 5-fold reduction in lyase activity compared with wild type. The defect in D44V appears to be in catalysis, since analysis by electrophoretic mobility shift (Fig. 3C) shows that EcoNth D44V efficiently bound to a substrate containing an AP site but failed to cleave it. These data substantiate that substitution of valine for aspartic acid greatly reduced the lyase activity of the enzyme and support the hypothesis that Asp-44 is important for the lyase activity of EcoNth. As expected, addition of NaOH to R184A reactions did not affect the amount of product produced for any substrates tested (Fig. 2B) showing that R184A retained its bifunctional character.

Enzyme Activity of Wild Type EcoNth and EcoNth R184A for Tg:A, Tg:G, DHU:G, or DHU:G—Wild type EcoNth or EcoNth R184A was incubated with substrates containing Tg:A, Tg:G, DHU:A, and DHU:G and the products analyzed by denaturing PAGE. The amount of nicked product produced was plotted as a function of enzyme concentration. Fig. 4A shows that under multiple turnover conditions and at low concentrations of enzyme, wild type EcoNth efficiently cleaved Tg:A, Tg:G, and DHU:G, but DHU:A was a much poorer substrate (see also Table TWO). Fig. 4B shows that for EcoNth R184A, under conditions of...
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**Figure 4.** Differential activities of EcoNth (A) and R184A (B) for Tg:A, Tg:G, DHU:A, and DHU:G. The percentage of nicked products was determined as described under "Materials and Methods." Closed circles, Tg:A; closed triangles, Tg:G; open circles, DHU:A; open triangles, DHU:G.

**Table Two**

| Substrate | $K_m$ (nM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ | $k_{cat}/K_m$ × 10$^6$ |
|-----------|-----------|------------------|---------------|------------------|
| EcoNth    |           |                  |               |                  |
| Tg:A      | 9.7       | 5.9              | 61            |                  |
| Tg:G      | 5.9       | 4.5              | 76            |                  |
| DHU:A     | 9.1       | 1.8              | 20            |                  |
| DHU:G     | 3.6       | 2.6              | 72            |                  |
| EcoNth R184A |       |                  |               |                  |
| Tg:A      | 4000      | 5.9              | 0.15          |                  |
| Tg:G      | 1920      | 96               | 5.0           |                  |
| DHU:A     | 140,000   | 82               | 0.06          |                  |
| DHU:G     | 133       | 10               | 7.5           |                  |

The formation of such covalent adducts has been demonstrated for EcoNth (35). To determine whether EcoNth S39L, D44V, and R184A were able to form a Schiff base, the enzymes were incubated with a substrate containing a DHU opposite G or an AP site opposite G in the presence or absence of NaCNBH$_3$ and the cross-linked products were analyzed by SDS-PAGE. Fig. 5 shows that EcoNth D44V and R184A (and EcoNth), but not S39L, formed covalent DNA-enzyme adducts with DHU-containing oligonucleotides. All three variants formed the Schiff base adducts with AP site-containing oligonucleotides in the presence of NaCNBH$_3$.

**DISCUSSION**

It is usual to informally classify amino acids in enzymes as having roles, if any, in reaction chemistry, enzyme structure, and substrate specificity. For example, in EcoNth Lys$^{120}$ is involved in reaction chemistry because it forms a Schiff base with the substrate, and lysines are observed at homologous positions in HhH-GPD superfAMILY enzymes having lyase activity. In contrast, EcoNth Gly$^{116}$ and Gly$^{118}$ are presumed to be important for helix-hairpin-helix structure and are highly conserved throughout the HhH-GPD superfAMILY. However, identification of amino acids having roles in specificity is much harder, and very little is known about the sequence basis of EcoNth specificity. The fundamental problem is in strategically selecting substitutions that will alter specificity without eliminating activity altogether. Since evolution of paralogous proteins is such a selection procedure, our approach is to take advantage of natural sequence phenologies of the HhH-GPD superfAMILY. This approach succeeds when few site-directed mutations result in either unchanged activities or complete loss of activity. In this study three out of three site-directed mutations exhibited functional differences that could be understood in the context of EcoNth structure.

What do the extant crystal structures tell us about the amino acids selected by phylogenetic analysis? In the B. stearothermophilus Nth-DNA complex (29), Ser$^{40}$, homologous to EcoNth Ser$^{39}$, does not appear to have any contacts with the DNA backbone, solvent, or the ring-opened sugar, but in the modeled active site, Ser$^{40}$ is positioned to interact with the damaged base (data not shown). The corresponding Leu$^{40}$ in MutY interacts with the adenine face in the MutY-DNA complex (36). The EcoNth S39L mutation studied here has lost its glycosylase activity while maintaining lyase activity (Figs. 2 and 3). The loss of glycosylase activity could be due to steric interference resulting from the larger size of leucine, the loss of a hydroxyl group, or both of these changes.

Fromme and Verdine (29) predicted that an Asp$^{45}$, homologous to Asp$^{44}$ in EcoNth, would play an essential role in Bacillus stearother-
mophilus Nth lyase activity by abstracting a hydrogen from the C2’ position of the damaged nucleotide, catalyzing β-elimination. Our data support the proposed role of Asp44 in catalysis, since EcoNth D44V both binds to (Fig. 3C) and forms a Schiff base (Fig. 5) with DNA containing an AP site. Furthermore, EcoNth D44V retains glycosylase activity but has greatly reduced lyase activity (Figs. 2 and 3). The x-ray structures of B. stearothermophilus Nth-DNA complexes (29) showed that Asp85 appears to hydrogen-bond a water molecule that is near the Pro-R proton at C2’. The authors (29) proposed that this water and Asp85 shuttle the Pro-R proton catalyzing β-elimination, consistent with their observation that B. stearothermophilus Nth removes the Pro-R proton with 97% selectivity. The corresponding Val35 in the MutY protein is in the minor groove reading frame of the protein. Notably, a MutY V45N mutation eliminates glycosylase activity (36). Guan et al. (36) postulate that MutY V45N loses activity because it disrupts adenine nucleotide flipping into the active site.

Although our results with D44V are in agreement with the prediction from Fromme and Verdine (29), they raise two questions. First, as these authors point out, it has been reported that EcoNth removes the Pro-S proton (37). Second, although MutY does not exhibit lyase activity, a site-directed mutant having a lysine homologous to EcoNth Lys120 does exhibit significant lyase activity in the absence of an aspartate homologous to EcoNth Asp84 (38). Vassylyev et al. (39) suggest that Glu23 of T4-endo V could participate in the lyase activity of this protein by abstracting the Pro-S hydrogen at the C2’ position. Correspondingly, in the MutY variant, S120K, abstraction of the hydrogen at the C2’ position may be mediated by Glu23, which is in the vicinity of the active site of MutY and is not conserved in EcoNth. Although the lyase activity of EcoNth D44V was substantially reduced compared with the wild type enzyme, D44V did exhibit a low level of lyase activity (Fig. 3). In this case, Glu23 of EcoNth D44V, which is in vicinity of the active site of EcoNth, might abstract the C2’ proton albeit with significantly lower efficiency.

In the structure of BstNth (29) Arg186, homologous to EcoNth Arg184, hydrogen-bonds to the 5’ oxygen of the AP site and to Glu24, homologous to EcoNth Glu23. EcoNth Arg184 is also positioned to interact with the damaged base (data not shown). Interestingly, R184A retains both glycosylase and lyase activity, but its interaction with substrates containing thymine glycol and dihydrouracil is different from that of wild type EcoNth. Arg184 appears to be critical for recognition or catalysis of substrates containing Tg or DHU paired with A because R184A cleaves these substrates poorly (Figs. 2A and 4B); however, R184A can cleave both lesions oppositely (Fig. 4B). Since Arg184 is not positioned to interact directly with the base opposite the damage, these results suggest that EcoNth Arg184 plays an indirect role in substrate specificity through a direct role in positioning/stabilizing the substrate via interaction with the 5’ oxygen of the damaged base. Nth orthologs have been shown to exhibit an opposite guanine preference (16, 40, 41), and the crystal structure of the Nth-DNA complex shows the enzyme to make more contacts with estranged G than estranged A (29). We propose that Arg184 partially compensates for relatively poor binding to adenine opposite a damaged base.

In summary, previous studies have focused on Lys120 and Asp138 as the key catalytic residues in Nth orthologs. Our results, in combination with the analysis provided from Fromme and Verdine (29), show that Asp44 plays an important role in the AP lyase step of EcoNth. Furthermore, we demonstrate that Ser39 is involved in base recognition or binding, while Arg184 also plays a role in substrate specificity and is possibly involved with opposite base specificity. This study illustrates the utility of “phylogenetic scans” in designing sets of site-directed mutations based on identification of sequence-function correlations in sets of homologous proteins to delineate the role of specific amino acids in enzyme substrate interactions.

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