Identification of a Zinc Finger Domain in the Human NEIL2 (Nei-like-2) Protein*

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The recently identified human NEIL2 (Nei-like-2) protein, a DNA glycosylase/AP lyase specific for oxidatively damaged bases, shares structural features and reaction mechanisms with the Escherichia coli DNA glycosylases, Nei and Fpg. Amino acid sequence analysis of NEIL2 suggested it to have a zinc finger-like Nei/Fpg. However, the Cys-X2-His-X10-Cys-X2-Cys (CHCC) motif present near the C terminus of NEIL2 is distinct from the zinc finger motifs of Nei/Fpg, which are of the C4 type. Here we show the presence of an equimolar amount of zinc in NEIL2 by inductively coupled plasma mass spectrometry. Individual mutations of Cys-291, His-295, Cys-315, and Cys-318, candidate residues for coordinating zinc, inactivated the enzyme by abolishing its DNA binding activity. H295A and C318S mutants were also shown to lack bound zinc, and a significant change in their secondary structure was revealed by CD spectra analysis. Molecular modeling revealed Arg-310 of NEIL2 to be a critical residue in its zinc binding pocket, which is highly conserved throughout the Fpg/Nei family. A R310Q mutation significantly reduced the activity of NEIL2. We therefore conclude that the zinc finger motif in NEIL2 is essential for its structural integrity and enzyme activity.

Oxidative DNA damage has been implicated in mutagenesis and is suggested to be involved in the etiology of aging and many diseases including cancer (1, 2). Repair of oxidatively damaged bases in all of the organisms occurs primarily via the DNA base excision repair pathway, which is initiated with the excision of damaged bases by DNA glycosylases (3). Until recently, only two DNA glycosylases, NTH1 (endonuclease III homolog) and OGG1 (8-oxoguanine DNA glycosylase), have been characterized in mammals, which are responsible for repair of oxidized pyrimidine and purine base lesions, respectively. Both OGG1 and NTH1, orthologs of the Escherichia coli glycosylase Nth, utilize an internal Lys residue as the active

site nucleophile and carry out β-elimination at the abasic (AP)¹ site generated after base removal (4, 5). However, E. coli has two other oxidized base-specific DNA glycosylases, namely MutM/Fpg and its paralog, Nei (6, 7), which utilize the N-terminal Pro as the active site nucleophile (8) and carry out β-elimination at the AP site after excising the base lesion. We and others (9–13) recently discovered and characterized two other mammalian DNA glycosylases and named these NEIL1 and NEIL2 (Nei-like-1 and -2), which are orthologs of E. coli Fpg/Nei (9–13). Both NEILs use the N-terminal Pro as the active site and function as a DNA glycosylase/AP lyase to carry out β-elimination (9, 10). The recombinant NEILs are active in excising a variety of oxidatively damaged bases but show significant differences in substrate preference. NEIL1 prefers reactive oxygen species-derived pyrimidine lesions and also efficiently removes FapyG and FapyA, the ring-opened oxidation products of purines (9, 12). NEIL2 removes oxidized pyrimidine substrates from duplex DNA but is more efficient in excising oxidized bases when they are located in a DNA bubble structure (14).

NEIL2 shares overall identity of 32 and 27% with Fpg and Nei, respectively, and the key residues of the E. coli enzymes, particularly the N-terminal PE(L/G)P(E/L) motif, are completely conserved in NEIL2 (10). In contrast to the Nth family, the Fpg/Nei family utilizes two DNA binding motifs, a helix-two-turn-helix (15) and a zinc finger motif (16). Fpg and Nei share significant homology with each other including the sequence of the zinc finger motif, which is of the C4 type (17). The zinc finger motifs are often involved in specific DNA recognition and have been identified in many DNA-binding proteins, transcription factors, and products of developmental control genes (18–21). Furthermore, several proteins associated with DNA repair, such as Xeroderma Pigmentosum complementation group A, poly(ADP-ribose) polymerase, and replication-associated protein A (RPA), have been shown to contain zinc finger domains (22–25). In E. coli, the Uvra protein, which is involved in DNA damage recognition during nucleotide excision repair, also possesses zinc finger domain (26, 27). Identification of zinc finger motifs in the ever-growing number of DNA-binding proteins is based primarily on the presence of conserved Cys or His residues and the spacing between them, which may be critical in recognition of specific double-stranded DNA sequences.

Here we show that NEIL2 possesses a single unusual CHCC-type zinc finger motif at its C terminus, which is distinct from

¹ The abbreviations used are: AP, abasic; DTT, dithiothreitol; Fpg, FapyG-DNA glycosylase; Nei, endonuclease VIII; NEIL, Nei-like; PBS, phosphate-buffered saline; WT, wild type; B11, 11-nt bubble; PDB, Protein Data Bank; 5-OHU-G, 5-hydroxyuracil.

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The atomic coordinates and structure factors (code 1vzp) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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that of Nei/Fpg, and that this motif is essential for maintaining the structural integrity and activity of NEIL2.

**EXPERIMENTAL PROCEDURES**

**Expression of Wild-type (WT) and Mutant NEIL2 Polypeptides**—The WT full-length NEIL2 was cloned between the NdeI/XhoI sites of the expression plasmid pSETB (Invitrogen) (10). The NEIL2 mutants (C291S, H295A, C315S, C318S, and R310A) were generated using a site-directed mutagenesis kit (Stratagene), and their authenticity was confirmed by direct DNA sequencing.

Log-phase cultures of *E. coli* DE884 mutM nei were transformed with expression plasmids of WT and mutant NEIL2 and then induced with 0.2 mM isopropyl-1-thio-D-galactopyranoside at 16 °C for 16 h. After centrifugation, the cell pellets were suspended in a lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 100 mg/ml lysozyme, 5 mM dithiothreitol (DTT), and protease inhibitor mixture. After sonication and centrifugation, the supernatant was used for Western blot analysis or activity assay by trapping analysis (10).

**Purification of Anti-NEIL2 Antibody and Western Blot Analysis**—Polyclonal anti-NEIL2 antibodies were purified from rabbit antisera produced by Alpha Diagnostics (San Antonio, TX) by affinity chromatography on Sepharose 4B (Amersham Biosciences) covalently coupled with NEIL2. NEIL2-specific IgG was eluted with glycine-HCl, pH 2.8, and confirmed by direct DNA sequencing. Polyclonal anti-NEIL2 antibodies were purified from rabbit antisera produced by Alpha Diagnostics (San Antonio, TX) by affinity chromatography on Sepharose 4B (Amersham Biosciences) covalently coupled with NEIL2. NEIL2-specific IgG was eluted with glycine-HCl, pH 2.8, and confirmed by direct DNA sequencing.

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**Incision Assay with 5-OHU-containing Bubble Oligomer**—DNA strand cleavage at the abasic (AP) site after damaged base excision by NEIL2 occurs because of its intrinsic AP lyase activity. We have shown previously that NEIL2 has higher activity when the lesion is inside a bubble in an otherwise duplex oligomer. The strand incision by NEIL2 was used for its assay using an oligomer containing 5-OHU in the middle of unpaired 11-nt bubble (B11) as described previously (14). The 51-mer oligomer, 32P-labeled at the 5′ terminus of the lesion-containing strand, was incubated with NEIL2 (WT and mutants) at 37 °C for 15 min in a 15-μl reaction mixture containing 40 mM Hepes, pH 7.5, 50 mM KCl, 100 μg/ml bovine serum albumin, and 5% glycerol. After the reaction was stopped with 80% formamide and 20 mM NaOH, the cleaved oligomers were separated by denaturing gel electrophoresis in 15% polyacrylamide containing 7 mM urea in 90 mM Tris borate, pH 8.3. Nei and Fpg were aligned with the C-terminal sequence of NEIL2. The position of the helix two-turn helix motif (H2TH) is indicated, and the coordinating amino acid residues forming the zinc finger motif were boxed. A, schematic diagram of the C terminus of NEIL2 bearing the putative zinc finger motif.

**FIG. 1. Putative zinc finger domain of NEIL2.** A, amino acid sequence alignment of NEIL2 with *E. coli* Nei and Fpg. The C termini of Nei and Fpg were aligned with the C-terminal sequence of NEIL2. The position of the helix two-turn helix motif (H2TH) is indicated, and the coordinating amino acid residues forming the zinc finger motif were boxed. B, schematic diagram of the C terminus of NEIL2 bearing the putative zinc finger motif.
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8.3, and 2 mM EDTA. The radioactivity in the substrate and cleaved product was analyzed by PhosphorImager (Amersham Biosciences).

Circular Dichroism Spectroscopy—All of the CD spectra were collected from proteins after dialysis and filtration in an AVIV 60DS spectrometer at 25 °C. An average of three scans of the spectra (250–200 nm) was used to obtain the final data. The molar ellipticity (θ) was calculated using Equation 1,

\[
\theta = \frac{\theta_{obs} \times 10^{-3} \times MW}{C \times l \times n \times 10^2} \text{deg dmol}^{-1} \text{cm}^2 (\text{Eq. 1})
\]

where \(\theta_{obs}\) is the observed ellipticity, MW is molecular weight, C is concentration (mg/ml), l is the path length of the cuvette in centimeters, and n refers to the number of residues. Protein concentrations were determined by the Bradford assay using bovine serum albumin as the standard.

Molecular Modeling of 192–319 Residues of NEIL2—The sequence of NEIL2 with the potential DNA binding region (residues 192–319) was used as the seed sequence to search for a suitable template using BIOSERVER (meta server located at bioserv.cbs.cnrs.fr/). BIOSERVER submits to fold-recognition servers like 3D-PSSM (28), mGenThreader (29), SAM-T99 (30), and (Protein Data Bank (PDB)-BLAST). The results from different servers are parsed automatically, and the TITO program is used to evaluate most compatible template (31). The crystal structure of E. coli formamidopyrimidine-DNA glycosylase (Fpg/MutM, PDB code 1rmd, residues Cys-41, His-43, Cys-61, Cys-64). Upper- and lower-bound dihedral angle constraints were defined by adding or subtracting 5°. A total of 30 distance constraints per atom were extracted from the matching regions of the template. Models were generated using the distance geometry program DIAMOD. A few cycles of constrained energy minimization were applied using the program FANTOM, which minimizes constraint energies by successive application of quasi-Newton and Newton-Raphson minimizers (36), using the ECEPP2 forcefield. The conformational energy of the model after energy minimization was −410 kcal/mol.

RESULTS

Expression and Activity of WT and Mutant NEIL2 in Crude E. coli Extracts—Sequence alignment of NEIL2 with Fpg and Nei predicted that NEIL2 is a zinc finger protein with a CHCC-type motif near the C terminus. Cys-291, His-295, Cys-315, and Cys-318 are candidate residues for coordinating Zn\(^{2+}\). A total of 30 distance constraints per atom were extracted from the matching regions of the template. Models were generated using the distance geometry program DIAMOD. A few cycles of constrained energy minimization were applied using the program FANTOM, which minimizes constraint energies by successive application of quasi-Newton and Newton-Raphson minimizers (36), using the ECEPP2 forcefield. The conformational energy of the model after energy minimization was −410 kcal/mol.

All of the DNA glycosylases/AP lyases, regardless of their
substrate preference, form transient Schiff bases with free AP site in DNA, which could be reduced with NaCNBH$_3$ (or NaBH$_4$) to form a stable "trapped complex" (37, 38). We have shown earlier that NEIL2, similar to other MutM/Nei type enzymes, is inactivated when the N-terminal Pro, the active site nucleophile, is blocked or eliminated (10). The WT and two mutant NEIL2 expression plasmids after incubation of a 5-OHU-containing oligomer. Because the mobility of such complexes reflects the size of the DNA glycosylase when the same oligomer substrate is used, it is evident that, in control E. coli lacking Fpg and Nei, only endogenous Nth formed a major trapped complex (lane 3). Crude bacterial lysates harboring WT NEIL2 formed a trapped complex of the same size as the purified recombinant NEIL2 (lane 2) used as a marker. However, the lack of such trapped complexes with crude lysates expressing various mutant NEIL2 proteins (lanes 5–8) suggests that the mutants are inactive as AP lyases. This loss of activity is probably the result of a loss of the zinc finger motif, critical either for structural integrity or the DNA binding activity of this enzyme.

**Table I**

| NEIL2 proteins | Mol of zinc/mol of NEIL2 |
|----------------|-------------------------|
| Wild type      | 0.97 ± 0.086            |
| C318S          | 0.15 ± 0.014            |
| H295A          | 0.08 ± 0.009            |

**Fig. 5.** CD spectra of purified WT and mutant NEIL2. Far-UV CD spectra of WT and mutant NEIL2 (H295A and C318S) in PBS (pH 7.0) at 25 °C.

**Structural Alterations in H295A and C318S NEIL2 Mutants**—The presence of zinc was shown to be essential for the folding and stability of many classical Zn$^{2+}$ finger proteins. Based on our results that Cys-291, His-295, Cys-315, and Cys-318 residues are responsible for coordinating Zn$^{2+}$, we examined the effect of mutation in these residues on the secondary structure of the protein by analyzing the CD spectra of purified WT and two mutant NEIL2 (H295A and C318S) proteins. The far-UV CD spectrum (Fig. 5) shows that WT NEIL2 has distinct secondary structure as indicated by the minima in molar ellipticity at 208 and 222 nm. However, the CD spectra of both the H295A and C318S proteins showed strong reduction in the mean residue ellipticity. These results indicate gross structural changes induced by mutations at His-295 or Cys-318 of NEIL2.

**Molecular Model of NEIL2 Residues 192–319**—A homology model of C-terminal residues 192–319 of NEIL2 was built using E. coli Fpg (PDB code 1k82) (32). The structural alignment used for the modeling is represented in Fig. 6A. The final model (Fig. 6B) showed a backbone root mean square deviation value of 0.55 Å to the template. The models consist of helices formed by residues 203–208, 216–220, 231–241, and 254–271, a 3$_{10}$-helix, and the zinc finger motifs starting from residues 231 and 254.
helix by residues 249–251, and a β-sheet consisting of two β-strands formed by residues 300–302 and 312–314. The model has helix-two turn-helix motif formed by four helices and a novel β-hairpin CHCC-type zinc finger. The CHCC-type zinc finger is formed by residues Cys-291, His-295, Cys-315, and Cys-318. Mutations in any one of these residues abolish the function of NEIL2. The β-hairpin zinc finger provides a necessary structural framework to position the conserved Arg-310 that may take part in the catalytic reaction. By superimposing the model on the co-crystal structure of DNA bound to E. coli Fpg (PDB code 1K82), we found that the conserved Arg-310 (Fig. 6C) is placed in a position similar to Arg-258 in E. coli Fpg (32). Thus our model of human NEIL2 provides a mechanistic insight into the tertiary structure of the protein. The model is available at www.rcsb.org (PDB code 1vzp).

**Requirement of Arg-310 for Enzyme Activity**—To clarify the role of the conserved Arg residue identified by molecular modeling (Fig. 6C), we constructed and characterized the NEIL2 site-directed mutant, R310Q. The purified R310Q protein showed greatly diminished activity relative to the WT NEIL2 with a 5-OHU containing bubble DNA substrate (Fig. 7A) and also decreased DNA binding (Fig. 7B). Thus these results further confirm that the zinc finger positions the conserved Arg-310 correctly in the active site pocket of NEIL2.

**DISCUSSION**

Zinc is an essential trace element and the second most abundant metal in mammalian cells. The adult human body contains $\sim 3 \text{ g}$ of $\text{Zn}^{2+}$ (39). It plays key roles in the maintenance of chromatin structure and also in nucleic acid metabolism as a structural component of enzymes in DNA replication, transcription, and DNA repair (40). The binding of zinc stabilizes the folded conformations of protein domains so that they may facilitate interactions with other macromolecules such as DNA. The lack of redox activity for the zinc ion and its binding and exchange kinetics may also be important in the use of zinc for specific functional roles, unlike other transition metal ions that might engage in free radical generation, leading to carcinogenic oxidative damage in cells (41). The peptide motifs containing bound zinc and named "zinc fingers" in proteins were first...
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identified approximately 20 years ago during the investigation of eukaryotic transcription factors. Since then, >10 different classes of zinc finger motifs have been discovered and characterized, many for their ability to bind nucleic acids in a sequence-specific manner and others for specifically mediating protein-protein interactions (42). Unlike the typical mode of Zn\(^{2+}\) coordination within the catalytic center of enzymes, tetrahedral coordination of Zn\(^{2+}\) in zinc fingers characteristically involves 2–4 potentially redox-reactive sulphydryl groups (cysteine). It is estimated that zinc finger proteins constitute up to 1% of all human gene products with each of these proteins containing from 1 to 30 repeats of cysteine (+ histidine)-containing zinc finger motifs.

NEIL1 and NEIL2 are two orthologs of E. coli Fpg/Nei, which were shown to be zinc finger proteins. Although NEIL1 and NEIL2 have significant functional overlap and use the same reaction chemistry as Fpg and Nei, only NEIL2 possesses a potential zinc finger motif. Near the C terminus, it contains a unique sequence with three Cys and one His residues in an unusual zinc finger configuration (Fig. 1) that is not homologous to the zinc finger motifs of Nei/Fpg. In this study, we set out to confirm that NEIL2 is indeed a zinc finger protein and then examine the role of this motif in the structure and function of this glycosylase.

We used site-directed mutagenesis to confirm the requirement for the three Cys and one His residues predicted to coordinate with zinc in the putative zinc finger domain of NEIL2. The DNA-trapping assay is a fast and definitive method for assessing the base excision activity of DNA glycosylase/AP lyases. Because traped complexes with a radiolabeled oligomer can be separated by SDS-PAGE that can identified whether any of the mutant proteins has DNA glycosylase/AP lyase activity. The absence of trapped complexes with all four mutants confirmed that mutation of any one of these Cys and His residues totally abolished the enzymatic activity (Fig. 2). This is consistent with a critical role for the zinc finger in enzymatic function, as was also observed for Fpg and Nei (44, 45). We then tested whether the loss of enzymatic activity was due to loss of DNA binding activity. We carried out electrophoretic mobility shift assay with purified WT and mutant proteins using a 5-OHU-containing bubble substrate oligomer. All of the cysteine mutants and the single histidine mutant failed to bind substrate DNA, indicating that the zinc finger motif is essential for DNA binding. Subsequent studies confirmed that the purified mutant NEIL2s have no strand incision activity with the substrate oligomer (Fig. 4). Finally, mass spectroscopic analysis showed that the WT NEIL2 contained 1 mol of zinc per mol of protein, whereas the mutants (C318S and H295A) lacked bound Zn\(^{2+}\) (Table I). This provided the strongest evidence that the candidate Cys and His residues are indeed responsible for zinc coordination and that the mutation of even one residue abolishes this coordination. This raised the question as to whether the loss of Zn\(^{2+}\) induces any change in the secondary structure of NEIL2. The CD spectrum clearly showed that the secondary structures of the C318S and H295A mutants were drastically altered (Fig. 5). Mutations in these residues are likely to prevent proper folding of the β-strands into a hairpin motif (Fig. 6B), and hence the geometry of the critical conserved residue Arg-310 (predicted from the model) may not be situated correctly. Mutation of Arg-310 strongly reduced NEIL2 activity without significantly affecting DNA binding (Fig. 7), unlike the mutations in zinc-coordinating residues. This strongly suggests that Arg-310 in NEIL2, positioned by the zinc binding pocket, is highly conserved as in the Fpg/Nei family and performs an indispensable catalytic role similar to that of Arg-258 in E. coli Fpg (32) or Arg-253 in E. coli Nei (46). In both cases, this residue participates in protonation of 5′-phosphate. It was recently shown that the conserved Arg is also present in the other mammalian nei homolog, NEIL1 (47). Although mammalian NEIL1 has similar folds as the bacterial Fpg/Nei, it has an unusual motif near the C terminus. This structural motif mimics the β-hairpin zinc finger found in members of the Fpg/Nei class (including NEIL2) but lacks the loops harboring the canonical zinc-binding residues and therefore does not coordinate zinc. Interestingly, the critical Arg (Arg-277 in NEIL1) is positioned in the loop connecting the two β-strands of the zinc-less finger and mutation to Ala showed a strong reduction of glycosylase activity (47). We have shown here that the R310Q mutant of NEIL2 also has markedly reduced glycosylase activity. Thus the conserved Arg is positioned critically for glycosylase activity in both the zinc-containing (NEIL2 and Fpg/Nei) and zinc-less fingers (NEIL1).

Thus our studies reveal that the localized destabilization of the zinc finger motif in NEIL2 affected the conformation of the whole polypeptide. The structural perturbation in the mutants was also reflected during purification. The WT NEIL2 binds strongly to the SP resin during fast protein liquid chromatography. In contrast, the zinc finger mutants did not bind to SP and bound weakly to the Q column. Taken together, our results provide definitive evidence for the identity of zinc-coordinating residues and show that the zinc finger motif is integral to the structure and function of NEIL2. We may note in passing that the nonconsensus (CHHC-type) motif is rather uncommon among the zinc finger proteins involved in DNA metabolism.

Further work with high resolution x-ray diffraction analysis will provide a more detailed understanding regarding specific residues involved in maintenance of the zinc finger domain and overall stability of the protein. A larger implication of this study involves public health issues such as malnutrition and hence lower zinc levels in the diet (48), or exposure to chemical toxins or radiation, which induce oxidative stress, could inactivate NEIL2, leading to reduced repair of oxidative damage of the genome.
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