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Crystallization and preliminary crystallographic analysis of endonuclease VIII in its uncomplexed form

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The *Escherichia coli* DNA repair enzyme endonuclease VIII (EndoVIII or Nei) excises oxidized pyrimidines from damaged DNA substrates. It overlaps in substrate specificity with endonuclease III and may serve as a back-up for this enzyme in *E. coli*. The three-dimensional structure of Nei covalently complexed with DNA has been recently determined, revealing the critical amino-acid residues required for DNA binding and catalytic activity. Based on this information, several site-specific mutants of the enzyme have been tested for activity against various substrates. Although the crystal structure of the DNA-bound enzyme has been fully determined, the important structure of the free enzyme has not previously been analyzed. In this report, the crystallization and preliminary crystallographic characterization of DNA-free Nei are described. Four different crystal habits are reported for wild-type Nei and two of its catalytic mutants. Despite being crystallized under different conditions, all habits belong to the same crystal form, with the same space group (*I*222) and a similar crystallographic unit cell (average parameters $a = 57.7$, $b = 80.2$, $c = 169.7$ Å). Two of these crystal habits, I and IV, appear to be suitable for full crystallographic analysis. Crystal habit I was obtained by vapour diffusion using PEG 8000, glycerol and calcium acetate. Crystal habit IV was obtained by a similar method using PEG 400 and magnesium chloride. Both crystals are mechanically strong and stable in the X-ray beam once frozen under cold nitrogen gas. A full diffraction data set has recently been collected from a wild-type Nei crystal of habit I (2.6 Å resolution, 85.2% completeness, $R_{\rm merge} = 9.8\%$). Additional diffraction data were collected from an Nei-R252A crystal of habit IV (2.05 Å resolution, 99.9% completeness, $R_{\rm merge} = 6.0\%$) and an Nei-E2A crystal of habit IV (2.25 Å resolution, 91.7% completeness, $R_{\rm merge} = 6.2\%$). These diffraction data were collected at 95–100 K using a synchrotron X-ray source and a CCD area detector. All three data sets are currently being used to obtain crystallographic phasing *via* molecular-replacement techniques.

1. Introduction

Free radicals and reactive oxygen species are constantly generated in cells as byproducts of aerobic cell metabolism. The most active of these is the hydroxyl radical, which can damage the nucleobases and the sugar units of DNA, leading to DNA lesions of several types or base loss. Such lesions lead to miscoding and genomic instability and may contribute to chronic diseases associated with aging and cancer. To prevent the harm inflicted by such lesions, cells have evolved a number of DNA repair mechanisms. These highly conserved repair enzymes deal with different types of DNA damage. One of the main pathways is base-excision repair (BER), which is initiated by the action of DNA glycosylases.

Nei (EC 3.2.2.–; endonuclease VIII) is a bifunctional BER enzyme with DNA glycosylase and AP lyase activities. *Escherichia coli* Nei is a monomeric protein containing 262 amino-acid residues with a molecular weight of 29.7 kDa. Nei is involved in the recognition and removal of oxidatively damaged pyrimidines, including thymine glycol (Tg), uracil glycol, dihydrothymine, dihydrouracil (DHU), 5-hydroxycytosine, 5-hydroxyuracil and β-ureidoisobutyric acid (Melamede *et al.*, 1994; Jiang *et al.*, 1997). Similar substrate preferences are reported for the bacterial DNA glycosylase Nth (endonuclease III), although the two

enzymes have no sequence homology. In contrast, Nei shares significant sequence and structural similarity with the bacterial DNA glycosylase Fpg (also known as MutM), whose primary substrates are 8-oxoguanine and formamidopyrimidine derivatives of purines embedded in duplex DNA.

Three mammalian Nei-like (Neil) homologues have recently been cloned and characterized. They have been identified in both humans (Bandaru *et al.*, 2002; Hazra *et al.*, 2002; Morland *et al.*, 2002) and mice (Takao *et al.*, 2002; Rosenquist *et al.*, 2003). Although these enzymes show only a relatively small sequence homology to bacterial Nei, there is a high degree of similarity in positions in the sequence that have previously been shown to be crucial for catalytic activity. The homologies and similarities to the human enzymes make Nei a relevant model system for understanding the mechanisms of DNA damage repair in mammalian cells.

We have recently established the three-dimensional structure of *E. coli* Nei (ecNei) covalently bound to a 13-mer double-stranded DNA substrate (Zharkov *et al.*, 2002). Structural analysis of this covalent complex was made possible by the reductive trapping of a Schiff-base intermediate of the catalytic reaction by sodium borohydride. This structure provided the first structural information concerning an Fpg family DNA glycosylase including its interactions with a DNA substrate. Moreover, this structural analysis provided important information concerning the catalytic action of Nei and especially the mechanistic details of β-elimination. The resulting Nei–DNA complex structure, determined at 1.25 Å resolution, demonstrated that Nei consists of two separate domains linked by a long polypeptide loop (Zharkov *et al.*, 2002). The N-terminal domain (amino acids 1–125) has an antiparallel β-sandwich fold, while the C-terminal domain (amino acids 135–262) consists of two DNA-binding motifs, the H2TH (helix–two turn–helix) motif and the zinc-finger motif. These two separate DNA-binding motifs interact with each other through a conserved lysine residue (Lys52), which is part of the N-terminal domain. The structure of this Nei–DNA complex and the specific enzyme–DNA interactions observed highlighted amino-acid residues of Nei that appeared to be of critical importance for binding and catalysis. These potential catalytic residues (*e.g.* Glu2, Glu5, Lys52 and Arg252) were subjected to site-directed mutagenesis and the mutants were tested for enzymatic activity (Zharkov *et al.*, 2002). These experiments clearly demonstrated that Glu2 and Arg252 are directly involved in the catalytic reaction.

Although the crystallization and crystal structure analysis of the Nei–DNA covalent complex were relatively straightforward, structural analysis of the Nei protein itself (without DNA) appeared to be more difficult than initially expected. The difficulties in the analysis were partly a consequence of the low solubility and the high flexibility of this protein in the absence of the stabilizing effect of the bound DNA.

In this report, we describe the crystallization of ecNei and the preliminary crystallographic characterization of the resulting ecNei crystals. It is shown that four crystal habits are generated from four different crystallization conditions using the wild-type enzyme and two of its catalytic mutants, E2A and R252A. Despite the different crystallization conditions and different morphologies, the four crystal habits are shown to belong to the same crystal form. Two of these crystal habits appear to be of sufficient quality and stability for a detailed high-resolution structural analysis of the Nei protein.

2. Experimental

2.1. Purification of ecNei

Wild-type (WT) ecNei protein was purified from an overexpressing strain of *E. coli* as previously described (Rieger *et al.*, 2000). The ecNei protein mutants (E2A and R252A) were produced using the QuikChange site-directed mutagenesis kit (Stratagene) and purified in a similar procedure. Each batch of the purified protein (WT and mutants) was subjected to the standard assay for Nei activity, following a previously described procedure (Zharkov *et al.*, 2002). Briefly, reaction mixtures included 50 n*M* ^{32}P-labelled oligonucleotide duplex containing a single abasic site residue and varying amounts of ecNei (total volume of 10 µl). The enzyme was diluted to working concentrations in a reaction buffer containing 0.5 mg ml^{-1} BSA. Reactions were initiated by adding the enzyme. The mixtures were then incubated for 5 min at 310 K and the reactions were stopped by mixing with 5 µl of formamide dye loading buffer and heating at 368 K for 1 min.

2.2. Crystallization experiments

Crystallization experiments were set up immediately after the last purification step of both WT and mutated ecNei. The purified protein was concentrated using Centricon centrifugal concentrators (Millipore, MA, USA) to approximately 4 mg ml^{-1}. All initial crystallization experiments were performed by the hanging-drop vapour-diffusion method using an extensive series of factorial screens (Jancarik & Kim, 1991). In general, these initial conditions were based on commercially available sets of screens. The WT and mutated ecNei protein crystallization drops were prepared by mixing the concentrated protein solution with an equal amount of each of the specific screen conditions to give a final drop volume of 4 µl. A similar crystallization procedure was reported for the covalent enzyme–DNA complexes of Nei and Fpg (Zharkov *et al.*, 2002; Gilboa *et al.*, 2002).

Each of these protein drops was suspended over a 1 ml reservoir solution in 4 × 6 VDX crystallization plates (Hampton Research, CA, USA) for a period of 14–60 d. Three different crystal habits were obtained from these initial experiments, one of which was found to be suitable for full data collection and crystallographic analysis (see below).

Additional crystallization experiments were performed in an attempt to crystallize ecNei catalytic mutants bound to their DNA substrate. The experiments were generally similar to those described above except that a DNA substrate was added to the protein drop with a protein:DNA ratio of 1:2 or 1:4. One of the conditions tested in these experiments resulted in crystals of different morphology (crystal habit IV) compared with those obtained in the previous conditions reported above. This crystal habit proved to be of suitable quality for a full crystallographic analysis, yet from the unit-cell parameters it was suspected that these crystals contained the protein alone.

Processing, reduction, indexing, integration and scaling of the diffraction data were conducted using the *DENZO* and *SCALEPACK* crystallographic programs (Otwinowski, 1993; Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Crystallization and characterization of WT ecNei

Attempts to crystallize WT ecNei resulted in three types of crystals (crystal habits I, II and III) differing from one another in the exact crystallization condition, the overall crystal morphology, the crystal quality and the crystal stability. Crystal habit I was obtained by mixing 2 µl of the fresh and concentrated protein solution with an equal volume of a precipitating solution containing 0.16 *M* calcium acetate, 0.08 *M*

Table 1
Representative crystallization characteristics of crystal habits I-IV of ecNei.

Crystal habit	I	II	III	IV
Nei protein	WT	R252A	WT	E2A, R252A
Protein concentration (mg ml^{-1})	4	4	4	4
Crystallization solution	0.16 *M* calcium acetate, 0.08 *M* sodium cacodylate buffer, 12–14%(*w*/*v*) PEG 8K, 20% glycerol	0.05 *M* cesium chloride, 0.1 *M* MES buffer, 30% Jeffamine M-600	0.17 *M* ammonium sulfate, 0.085 *M* cacodylate buffer, 25.5%(*w*/*v*) PEG 8K, 15% glycerol	25–35% PEG 400, 0.1 *M* Tris buffer, 0.2 *M* magnesium chloride
pH	6.5	6.5	6.5	8.0–8.4
No. crystals in drop	2–5	3	3	6–9
Growth period (d)	14–60	30–60	60	5–7
Crystal dimensions (mm)	0.05 × 0.02 × 0.01	0.07 × 0.03 × 0.02	0.05 × 0.02 × 0.01	0.3 × 0.15 × 0.1

sodium cacodylate buffer pH 6.5, 12–14%(*w*/*v*) polyethylene glycol (PEG) 8000 and 20% glycerol. Crystal habit II was obtained using a similar procedure with a precipitating solution containing 0.05 *M* cesium chloride, 0.1 *M* MES buffer pH 6.5 and 30% Jeffamine M-600. Crystal habit III was obtained using a similar procedure with a precipitating solution containing 0.17 *M* ammonium sulfate, 0.085 *M* sodium cacodylate buffer at pH 6.5, 25.5% PEG 8000 and 15% glycerol. For all three habits, the protein solution drops (containing protein: precipitating solution ratio of 1:1) were suspended over 1 ml of the corresponding precipitating solution. Crystals of habits I and II appeared after about two weeks and grew to their full size in 4–6 weeks (2–5 crystals in each drop). Crystals of habit III appeared after about two months and grew to their full size in another 2–3 months. Representative crystallization parameters of the three habits of WT Nei are listed in Table 1.

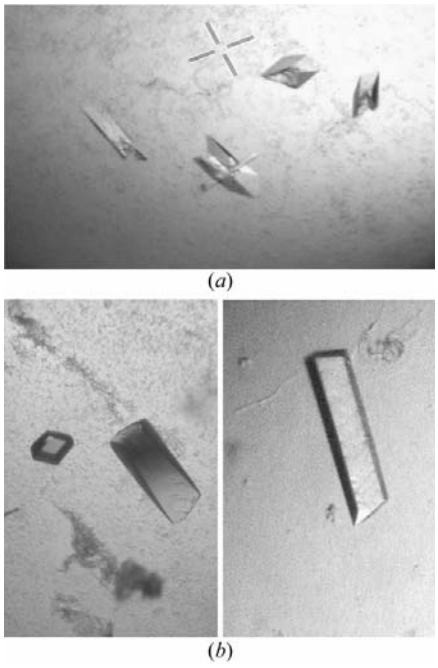

Figure 1
Typical single crystals of DNA-free Nei in the various orthorhombic crystal habits (space group *I*222). (*a*) Crystals of habit II that were used for the diffraction data measurement of WT ecNei at 3.15 Å resolution (typical crystal dimensions are about 0.07 × 0.03 × 0.02 mm). (*b*) Typical habit IV crystals of Nei-R252A (right) and Nei-E2A (left). Similar crystals were used for the diffraction data measurement of Nei-R252A at 2.05 Å resolution and Nei-E2A at 2.25 Å resolution. The bottom pictures were taken under polarized light. Crystal dimensions are about 0.4 × 0.1 × 0.06 mm (right) and 0.3 × 0.2 × 0.1 mm (large crystal, left).

Table 2
Selected crystallographic parameters for crystals of ecNei (WT and mutants).

Crystal habit	I	II	III	IV	IV
Specific protein	WT	R252A	WT	R252A	E2A
Crystal system	*I*-centered orthorhombic	*I*-centered orthorhombic	*I*-centered orthorhombic	*I*-centered orthorhombic	*I*-centered orthorhombic
Unit-cell parameters					
a (Å)	57.72	57.67	57.69	57.72	57.78
b (Å)	79.58	81.68	78.42	80.05	81.07
c (Å)	169.65	170.65	169.09	169.31	169.93
Space group	*I*222	*I*222	*I*222	*I*222	*I*222
Oscillation angle per frame (°)	0.5	0.5	0.5	0.5	0.5
Exposure time per frame (s)	40	10	30	10	10
No. frames	150	240	200	280	260
Data-collection temperature (K)	95	100	95	100	100
Resolution (Å)	2.60	3.15	3.55	2.05	2.25
No. reflections	156430	29733	17493	412998	376381
No. unique reflections	10629	7182	4825	25032	17458
Completeness (%)	85.2	97.1	85	99.9	91.7
$R_{\rm merge}$ (%)	9.8	7.6	10.7	6.0	6.2

Crystals of habit I appear usually as small rectangular parallelepipeds with sharp faces and edges and with typical dimensions of 0.05 × 0.02 × 0.01 mm. Initial characterization and a full crystallographic diffraction data measurement were performed at the X25 beamline of the National Synchrotron Light Source (NSLS) facility, Brookhaven National Laboratory (BNL, NY, USA). The diffraction data were obtained using a B4 CCD area detector (Brandeis University, USA) on one crystal flash-cooled in a cold nitrogen-gas stream (95 K). A series of 150 oscillation frames (40 s exposure; 0.5° oscillation) were collected, reduced, integrated and scaled together (using *DENZO* and *SCALEPACK*) to give a full data set extending to a 2.6 Å resolution limit. The diffraction pattern indicated that these crystals belong to the *I*-centred orthorhombic crystal system (space group *I*222), with unit-cell parameters *a* = 57.72, *b* = 79.58, *c* = 169.65 Å. A total of 156 430 accepted reflections were measured in the 40.0–2.60 Å resolution range, resulting in 10 629 unique reflections (85.2% completeness to 2.6 Å resolution, $R_{\rm merge}$ = 9.8%).

Crystals of habit II appear usually as elongated polygons in which one of the polygon faces is split into two parts (Fig. 1*a*). The typical dimensions of these crystals are around 0.07 × 0.03 × 0.02 mm (Table 1). Crystallographic characterization of this crystal habit was performed using a MAR CCD 165 detector (165 mm; MAR Research, Germany) at the 5-ID-B beamline of the Advanced Photon Source synchrotron facility (APS, Argonne National Laboratory, USA). These crystals diffracted to about 3.15 Å resolution and also belong to space group *I*222, with similar unit-cell parameters (*a* = 57.67, *b* = 81.68, *c* = 170.65 Å).

Crystals of habit III appear generally as much smaller elongated parallelepipeds, with typical dimensions of around 0.05 × 0.01 × 0.01 mm. As for habit I, these crystals were characterized using the B4 CCD area detector at the X25 NSLS beamline. These crystals diffracted to about 3.55 Å resolution and their diffraction pattern confirmed that

they also belong to space group *I*222. The unit-cell parameters are similar to habits I and II and average around a = 57.69, b = 78.42, c = 169.09 Å. A more detailed list of crystal habit characteristics is given in Table 2.

3.2. Crystallization and characterization of ecNei mutants

As noted above, attempts were made to crystallize two site-directed ecNei mutants together with their DNA substrate. Although these experiments did not produce the desired complexes of inactive Nei with DNA, one of the conditions tested resulted in crystals of habit IV. This condition was further refined and optimized to yield crystals of three specific Nei mutants, which appeared to be of better diffraction quality than the WT enzyme (in habits I–III). All attempts to obtain crystals of WT Nei under similar conditions failed, even when seeding procedures were applied.

The final procedure for the crystallization of the three ecNei mutants in crystal habit IV was as follows. EcNei mutant proteins (either E2A, E2Q or R252A) were purified as previously described (Zharkov *et al.*, 2002). 1.5 µl concentrated protein solution (4 mg ml^{-1}) was mixed with 2 µl crystallization solution (25–35% PEG 400, 0.1 *M* Tris–HCl buffer pH 8.0–8.4 and 0.2 *M* magnesium chloride). The 3.5 µl drop was then mixed with 0.5 µl of a solution containing double-stranded 13-mer oligonucleotide, with either Tg or DHU in position 7 of one of the strands. Drops were then suspended over 1 ml crystallization solution at 288 K. The crystals appeared in several days and grew to their full size in one week (Fig. 1*b*). The fully grown crystals of habit IV usually take the shape of elongated rectangular boxes. They are significantly larger than those of habits I, II and III, with typical dimensions of around 0.3 × 0.15 × 0.1 mm.

Initial crystallographic characterization and full data collection was performed on habit IV crystals of two Nei mutants, Nei-E2A and Nei-R252A. These diffraction measurements (10 s exposure and 0.5° oscillations to give a total of 90°) were performed using a MAR CCD (165 mm) area detector at the 5-ID-B APS beamline. Both crystals were found to belong to the *I*-centred space group *I*222, with unit-cell parameters very similar to those of crystal habits I, II and III. These similarities were unexpected owing to the significantly different crystallization conditions utilized to obtain these crystal habits, probably indicating highly favourable crystal packing in the unit cells.

A diffraction data set at 2.05 Å resolution was measured for Nei-R252A from a frozen crystal (100 K) of about 0.3 × 0.15 × 0.05 mm in size. A total of 412 998 accepted reflections [$F > 1.0\sigma(F)$] were measured, indexed, processed and merged, resulting in 25 032 independent reflections with 99.9% total completeness (100.0% completeness for the highest resolution shell, 2.09–2.05 Å), a total $R_{\rm merge}$ of 6.0% and an overall mosaicity of 0.6°. The refined specific unit-cell parameters of this crystal were a = 57.72, b = 80.05, c = 169.31 Å.

A second diffraction data set at 2.25 Å resolution was measured for Nei-E2A from a frozen crystal (100 K) of about 0.3 × 0.2 × 0.1 mm in size. A total of 376 381 accepted reflections [$F > 1.0\sigma(F)$] were measured, indexed, processed and merged, resulting in 17 458 independent reflections with 91.7% total completeness (88.2% completeness for the highest resolution shell, 2.29–2.25 Å), a total $R_{\rm merge}$ of 6.2% and an overall mosaicity of 1.1°. The specific unit-cell parameters of this crystal were a = 57.78, b = 81.07, c = 169.93 Å. Additional data-collection parameters are listed in Table 2.

3.3. Content of the unit cell

Since the space group and unit-cell parameters of all four crystal habits are virtually identical, it is safe to assume that all of them belong to an identical crystal form, having the same asymmetric unit and the same overall unit-cell content. Accordingly, all of the crystals reported above appear to contain the Nei protein only (WT or mutant), even in those cases where DNA was present during the crystallization process. Since the DNA substrate is relatively large (molecular weight of about 9 kDa) in comparison to the protein (molecular weight of about 30 kDa), it would have been expected to change the dimensions of the repeating unit in the crystal and hence the dimensions and volume of the unit cell. To varify these conclusions, crystals were washed and dissolved and the resulting solution was subjected to analytical DNA detection. No significant DNA content was found in any of the tested crystals. These observations also correlate well with the finding that the crystallographic unit cell of all four crystal habits is very similar despite the significantly different crystallization conditions used. As discussed above, these observations indicate that the molecular packing within this unit cell is likely to be highly favourable and that it pushes the equilibrium towards the side of its protein and DNA components. It appears that such favourable packing dominates the crystallization of Nei even with DNA substrates of relatively high affinity (with $K_{\rm d}$ values in the micromolar range). This picture is obviously different (and hence a totally different unit cell and packing) in the case of a covalent Nei–DNA cross-link, where such an equilibrium is not possible (Zharkov *et al.*, 2002).

The volume of the crystallographic unit cell of all the habits is also very similar. The average cell volume, as determined from the mean values of the unit-cell parameters at 100 K, is 7.82 × 10^5 Å^3. Assuming that the unit cell contains eight molecules (the minimal number possible, equalling the number of symmetry operations in space group *I*222) of the expressed monomer of native Nei (262 amino acids; MW = 29 714 Da), the calculated specific ratio of volume to protein in the crystal ($V_{\rm M}$) is 3.26 Å^3 Da^{-1}. This value, although relatively high, is within the normal range of $V_{\rm M}$ values observed for soluble protein crystals (1.7–3.5 Å^3 Da^{-1}; Matthews, 1968; Drenth, 1994). With this minimal number of Nei monomers in the unit cell, the calculated solvent content of the crystals is approximately 62%, a value that is again relatively high but reasonable. The next theoretical number of monomers in the unit cell is 16 (two in the asymmetric unit), which gives a $V_{\rm M}$ of 1.63 Å^3 Da^{-1} and solvent content of about 24%. Both values are outside of the common range and indicate that such a content of the unit cell is less likely. The present crystallographic data is therefore highly indicative of the presence of eight monomers of ecNei in the *I*222 unit cell and hence one monomer per crystallographic asymmetric unit. Current efforts to solve the crystallographic phase problem for these crystals are concentrated on molecular-replacement techniques.

4. Further studies

Of the four crystal habits described above, the most suitable for a complete crystallographic structural analysis is habit IV. Crystals of this habit are larger, more stable in the X-ray beam (once frozen) and grow significantly faster than the other crystal habits. They were shown to diffract to a reasonable resolution (2.3 Å and better), allowing a full crystal structure determination and meaningful structural analysis. Therefore, these crystals will be used to analyze the catalytic mutants of the DNA-free enzymes, Nei-E2A and Nei-R252A. Unfortunately, crystals of habit IV were

obtained only for mutated ecNei and efforts to obtain such crystals for the WT Nei have so far proved unsuccessful. The structural analysis of WT Nei will have to be performed with habit I crystals, as they diffract to the highest resolution (2.6 Å) and show the highest radiation stability of the three relevant habits.

Since all crystal habits belong to the same space group with similar unit-cell parameters, it is likely that the overall structure of the wild-type enzyme and its mutants do not differ significantly, except for possible small and local conformational changes. We will therefore start the structure-determination efforts of the DNA-free Nei with the highest resolution data of the Nei-R252A crystals. Once completed, the resulting model will be used as a starting point for the solution of the other structures (Nei-E2A and WT Nei). The initial structural analysis of the Nei-R252A crystals can be accomplished by molecular replacement, assuming that the general fold of the free Nei does not differ substantially from those already reported for members of the Fpg/Nei family. Two such reference models for molecular-replacement calculations could be the structure of the ecNei protein model from the ecNei–DNA complex structure (PDB code 1k3w; Zharkov *et al.*, 2002) or the DNA-free structure of the closely homologous *Thermus thermophilus* Fpg (ttFpg or ttMutM; PDB code 1ee8; Sugahara *et al.*, 2000). If successful, these molecular-replacement calculations are expected to provide initial phases and initial model of the ecNei-R252A structure, which could then be refined against the 2.05 Å resolution data described above. At later stages of this study, it is expected that the final structure of ecNei-R252A will be used for the full determination of the other DNA-free Nei structures, such as ecNei-E2A and WT Nei.

The expected high-resolution crystal structure of ecNei (and its mutants) will provide the first structure of any Nei protein and the second Fpg/Nei family protein for which the three-dimensional structure has been determined in the DNA-free form. This important structural data will allow us to study common features and differences among DNA glycosylases in general and among the Fpg family enzymes in particular. In addition, Nei will be the first enzyme of the Fpg/Nei family for which both DNA-bound and DNA-free forms obtained from the same biological source have been crystallized and analyzed. Comparing these two forms should enable a close examination of structural changes of the protein upon DNA binding, if any. Such a study is also expected to provide important information regarding the yet unresolved aspects of the catalytic mechanism of Nei, its non-specific binding to DNA and the mode of its release at the end of the catalytic cycle.

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