It was previously shown that 1,N⁶-ethenoadenine (eA) in DNA rearranges into a pyrimidine ring-opened derivative of 20-fold higher mutagenic potency in Escherichia coli (AB1157 lacΔU169) than the parental eA (Basu, A. K., Wood, M. L., Niedernhofer, L. J., Ramos, L. A., and Essigmann, J. M. (1993) Biochemistry 32, 12793–12801). We have found that at pH 7.0, the stability of the N-glycosidic bond in eA is 20-fold lower than in dA. In alkaline conditions, but also at neutrality, eA depurinates or converts into products: eA → B → C → D. Compound B is a product of water molecule addition to the C(2)–N(3) bond, which is in equilibrium with a product of N(1)–C(2) bond rupture in eA. Compound C is a deformed derivative of ring-opened compound B, which further depurinates yielding compound D. Ethenoadenine degradation products are not recognized by human N-alkylpurine-DNA glycosylase, which repairs eA. Product B is excised from oligodeoxynucleotides by E. coli formamidopyrimidine-DNA glycosylase (Fpg) and endonuclease III (Nth). Repair by the Fpg protein is as efficient as that of 7,8-dihydro-8-oxoguanine when the excised base is paired with dT and dC but is less favorable when paired with dG and dA. Ethenoadenine rearrangement products are formed in oligodeoxynucleotides also at neutral pH at the rate of about 2–3% per week at 37 °C, and therefore they may contribute to eA mutations.

1,N⁶-Ethenoadenine (eA)¹ and other exocyclic DNA adducts such as 3,N⁷-ethenocytosine (eC) or N⁷,3-ethenoguanine (eG) are introduced to DNA by the carcinogen vinyl chloride and related compounds (1). These DNA lesions are also formed during interaction with DNA of the peroxidation products of ω-6-polyunsaturated fatty acids (2). Ethenoadenine has been found in the DNA of unexposed humans and rodents at highly variable levels, ranging from 0.043 to 31.2 eA molecules/10⁶ of unmodified adenine residues (3, 4). Upon treatment of animals with vinyl chloride, the level of eA increased in the DNA of rat liver, lung, lymphocytes, and testis (in liver and lung several-fold) (3). The level of e-DNA adducts correlates with increased oxidative stress, such as observed during the accumulation of transient metal ions in Wilson disease, a human metal storage disease, and with increased content of polyunsaturated fatty acids in the diet (5–7).

Ethenoadenine is eliminated from DNA by N-methylpurine-DNA glycosylases. Eukaryotic glycosylases from yeast, rat, and human excise this lesion about 500-fold more efficiently than bacterial AlkA protein (8). Molecular dosimetry experiments suggest, however, that it is a persistent lesion, because 2 weeks after exposure of rats to vinyl chloride, the eA level in liver DNA remains very similar to that obtained directly after treatment (9).

All known exocyclic DNA adducts are mutagenic. In bacteria, eA is recognized mostly as an unmodified adenine by DNA polymerases, infrequently giving rise to AT → TA substitutions (10). In mammalian cells, e-DNA adducts are classified among lesions with the highest mutagenic potency. In site-directed mutagenesis either 70 (10) or 10% (11) of eA residues in DNA were replicated erroneously, giving rise mainly to AT → GC (10) but also to AT → TA (preferential mutation on the leading strand) and AT → CG substitutions (11). In the same studies only 0.3% of 7,8-dihydro-8-oxoguanine (8-oxG) residues induced GC → TA transversions (11). Treatment of mammalian cells with compounds inducing etheno-DNA adducts additionally triggers chromosomal aberrations and sister chromatid exchanges (1).

Early investigations by Tsou et al. (12) and Basu et al. (13, 14) have shown that in alkali, but also under physiological conditions, eA is rearranged into a pyrimidine ring-opened derivative, 4-amino-5-(imidazol-2-yl)imidazole, which has about 20-fold higher mutagenic potency in Escherichia coli (AB1157 lacΔU169) than the parental eA (14). Because the secondary lesions arising from eA might contribute significantly to its mutagenesis, we undertook a detailed study of the chemical stability of eA in DNA, during which we found enzymes repairing a derivative formed during eA chemical rearrangement and identified excised lesion.

EXPERIMENTAL PROCEDURES

Materials—1,N⁶-eA was synthesized using the modified procedure of Barrio et al. (15) described for the synthesis of its ribo-cogener. The material obtained (needles, melting point, 163–164 °C) was 99% pure (HPLC). Its identity was confirmed by UV, NMR, and electrospray MS. Oligodeoxynucleotide (40-mer) containing a single eA at position 20 in the sequence 5′-d(GCT ACC TAC CTA GCG ACC T eAC GAC TGT
Ring-opened Ethenoadenine Is Repaired by Fpg and Nth Protein

The solutions of dA (1–2 mM) were incubated in 0.2 N NaOH for 1–4 h, neutralized with the addition of an equivalent amount of 1 N HCl and 1/10 volume of 1 N Tris-HCl, pH 7.0, ethanol-precipitated, and washed. Then oligomers were digested enzymatically to nucleosides. The reaction mixture (50 μl) contained 1.5 nmol of oligomer, 1.5 units of nuclease P1, 0.67 units of snake venom phosphodiesterase, 0.3 units of E. coli alkaline phosphatase from Sigma.

**Instrumentation—**HPLC was performed using a Waters dual pump system with a tunable UV/visible light absorbance detector managed by Microcal Origin. Values obtained according to standard procedures using a Beckman oligo 1000 n synthesizer (Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences). E. coli Fpg and Nth proteins were purified from overproducing strains (JM105 carrying the pPGC230 plasmid and BH410 (as JM105 but fpg-1 K. Nakanishi carrying the pNTH10) as previously described (16, 17). Human N-methylpurine-DNA glycosylase (ANPG-40) as well as bacterial strains overproducing Fpg and Nth glycosylases were a kind gift from Dr. Jacques Laval (Institut G. Roussy, Villejuif, France). T4 kinase was from TaKaRa, nuclease P1 from Amersham Pharmacia Biotech, snake venom phosphodiesterase from PL Biochemicals, and E. coli alkaline phosphatase from Sigma.

**Thin Layer Chromatography—**Silica gel 60 F254 aluminum sheets (Merck, catalog no. 1.0554) were used for analytical purposes. For preparative purposes 20 × 20 cm plates were prepared using silica gel 60 PF254 (Merck, catalog no. 1.07747). The following solvent systems were used: methanol/chloroform, 1:1 (II); isopropyl alcohol/25% aqueous NH3/water, 70:10 (II) and 70:5:5 (III).

**Solvolytic Degradation of dA—**The solutions of dA (1–2 mM) were incubated in 0.5% NaOH (Varian) spectrometer equipped with a gradient generator unit Perform II, Ultrashirms, and a high stability temperature unit using 5 mm (Varian) spectrometer equipped with a gradient generator unit Perfom II, Ultrashirms, and a high stability temperature unit using 5 mm (Varian) spectrometer equipped with a gradient generator unit. The measured molecular mass of dA-oligomer was confirmed by mass spectrometry of nucleosides was performed on a Mariner apparatus with time of flight detection. UV spectra were recorded on a Cary 3E xen-ray film, scanned in an LKB densitometer, and quantified using Microcal Origin.

**Kinetic Studies with Determination of Km and Vmax for the Fpg Protein—**Kinetic constants were established in dA-oligomer incubated in 0.2 × 0.9 N NaOH for 4 h. The concentration range of the oligomer was 0.6–48 nM when the modified base was paired with dT, 4.8–65 nM when paired with dA, 0.3–75 nM paired with dC, and 2–160 nM paired with dG. The amounts of Fpg protein used in the reaction were adjusted to obtain less than 50% utilization of substrate and equaled 0.4 mg when modified base was paired with dT and 2.4 μg when paired with dA, and 0.2 mg with dC. In each experiment two control samples were set: negative with enzyme, to detect nonspecific breakage of oligodeoxynucleotide; and positive with excess enzyme (150 μg of Fpg), to get 100% cleavage of oligomer. The reaction was performed precisely for 10 min and stopped by adding sequencing kit stop solution, and reaction products were separated by PAGE. Autoradiograms were scanned (LKB scanner), and the peaks on resulting plots, corresponding to cleavage product and nonreacted oligomer, were quantified using multi-peak fitting in Microcal Origin. In calculations the average substrate concentration (i.e. ([S]1 + [S]2)/2) and average velocity (i.e. ([S]1 − [S]2)/2τ) were used (29). Vmax and Km values were calculated by two methods: a program using the Eisenhal-Cornish-Bowden nonparametric algorithm (30) and direct fitting of the hyperbolic Michaelis-Menten equation to the data points in Microcal Origin. Values obtained by both methods differed usually by no more than 5–6%.

**RESULTS**

alkali-induced Rearrangements of 1,6'-Ethenoadenosine—1,6'-Ethenoadenosine at pH 12 was degraded sequentially into three products: eDA → B → C → D (Fig. 1), which could be separated by HPLC (Fig. 2, A and B) or by TLC. The first stage, eDA → B, was the fastest (τb, at 23 °C was 2.5 h). The B → C and C → D reactions at pH 12 were at least 10 times slower than the conversion eDA → B (Fig. 2). The rearrangement of eDA → B is strongly pH-dependent; a half-time of this reaction at 37 °C equals 1.5 h at pH 12, 7 days at pH 9.2, while...
at pH 7.5 about 1 year, as estimated on the basis of 30-day measurements (not shown). A half-time for $B \rightarrow C$ and $C \rightarrow D$ reactions at pH 7.5, 37°C is about 12 days, as judged by the HPLC analysis of the isolated compounds $B$ and $C$. Thus, at physiological pH the first step of rearrangement in nucleosides occurs very slowly, and the subsequent steps are much faster.

We were searching for other than high pH factors that could stimulate edA rearrangements under physiological conditions. The degradation of edA was tested in the presence of several amino acids (glycine, l-proline, l-lysine, l-serine, all at 80 mM, and 80% saturated DL-tyrosine), 50 mM glutathione (reduced form), and 80 mM mercaptoethanol, as well as 100 mM NaHS, NaN$_3$, KF, and KI, all at 37°C in 0.1 M phosphate, pH 7.5. None of these compounds accelerated edA $\rightarrow$ B conversion (not shown).

**FIG. 1.** Proposed fate of 1,N$^6$-ethenodeoxyadenosine in DNA including structures of the parent edA and products of its degradation. The numbering of atoms as in the purine ring was retained in all structures for simplicity and convenience.

**FIG. 2.** Products of 1,N$^6$-ethenodeoxyadenine rearrangement in alkali. Panels A and B show HPLC separation of edA and products of its degradation formed at 23°C after 3 h (A) or 4 days (B) in 0.02 M NaOH. Chromatographic conditions were the following: linear gradient of 20 mM NH$_4$HCO$_3$, pH 9.0, 90% methanol in water over 60 min with a flow rate of 1 ml/min and UV absorbance detection at 260 nm. Panel C, kinetics of edA rearrangement in monomer (pH 12 at 23°C) as measured from the peak areas obtained in HPLC. Panel D, kinetics of edA rearrangement in polymer (pH 13 at 37°C) as measured from peak areas obtained in HPLC analysis and as described in Fig. 5.

**FIG. 3.** Part of the TOCSY spectrum of the product B in Me$_2$SO-$d_6$. Two traces corresponding to the sugar moieties of both isomers B1 and B2 are indicated. The two cross-peaks observed at 5.92/5.74 and 5.74/5.92 ppm corresponding to H$_1$ protons are due to B1 $\leftrightarrow$ B2 equilibrium.
Depurination of edA at Neutrality—HPLC analysis of neutral edA and dA depurination at 60 °C shows that the initial rate of edA formation is 0.08%/h, whereas that of A is 0.004%/h. Depurination of edA is concomitant with formation of product B with a rate of 0.2%/h (not shown); this shows that the glycosyl bond in edA is 20-fold less stable than that in dA and that the pyrimidine ring opening in edA is 2.5 times faster than the rupture of the glycosyl bond under neutral conditions.

Degradation of Ethenoadenine in Oligodeoxynucleotides—Composition of edA-oligomer incubated for 1–16 h in NaOH at 37 °C was analyzed by HPLC. Only edA, products B and C, could be identified, because compound D was masked by components of the buffer used for enzymatic digestion of oligomer (Figs. 2B and 5D). To reach a rate of edA → B conversion similar to that in nucleoside, oligomer was treated with NaOH at a concentration 10-fold higher than that used for nucleoside. Similarly, the fluorescence loss, because of decomposition of edA (the only fluorescent component of the pathway) was observed at a 10-fold higher concentration of NaOH in polymer than in monomer (not shown). Compounds B and C were already found in edA-oligomer not treated with NaOH (Fig. 2D), although their amount was negligible and differed from batch to batch; usually they constituted 2–6% of the expected edA amount (not shown). After 4 h of oligomer incubation in 0.2 N NaOH, edA B and C were found in comparable amounts (Fig. 2D), whereas a 4-h incubation of monomer in 0.02 N NaOH resulted in the conversion of 80% of edA into B (Fig. 2C). This suggests that in polymer the reaction is shifted toward the formation of compound C under conditions the same as those in the monomer rate of edA → B conversion.

Structure of Ethenodeoxyadenosine Products: NMR and MS Analysis—Proton chemical shifts of edA compounds B and C were assigned using TOCSY and ROESY; the spectra are listed in supplemental Table 1S and carbon chemical shifts in supplemental Table 2S.

The NMR signals in the spectra of edA have been assigned
Ring-opened Ethenoadenine Is Repaired by Fpg and Nth Protein

FIG. 5. HPLC identification of εA derivatives excised by Fpg and Nth proteins. εA-oligomer was incubated for 4 h in 0.2 N NaOH at 37 °C, treated or not with the excess of DNA repair glycosylases, and precipitated to remove excised base. Precipitated oligomer was digested to the nucleosides, which were separated subsequently by HPLC using isocratic elution with 20 mM NH₄HCO₃, pH 6.0, for 10 min and then a linear gradient of 20 mM NH₄HCO₃, pH 6.0, 30% methanol in water over 30 min at a flow rate 1 ml/min and UV absorbance detection at 260 nm. εA-oligomer incubated for 4 h in 0.2 N NaOH; B, the same as in A but digested with the Fpg protein; C, the same as in A but digested with the Nth protein; D, background signals derived from enzymatic solutions used for preparation of samples for HPLC. The relative quantity of εA (peak areas [Aₚₐₜ /εA]) in εA-oligomer untreated and treated with Fpg and Nth glycosylases was 12.9 (A), 15.9 (B), and 15.6 (C), whereas the quantity of compound B equaled 8.1 (A), 3.7 (B), and 3.4 (C). The quantity of compound C was 19.3 (A), 18.3 (B), and 18.3 (C).

TABLE I

| Substrate | Kₘ | kₘₐₓ | kₘₐₓ/Kₘ |
|-----------|----|------|---------|
| εA derivative paired with: | nm | min⁻¹ |         |
| dA        | 59.9 ± 20.5 | 0.49 ± 0.1 | 0.008   |
| dC        | 6.2 ± 1.1   | 0.42 ± 0.02 | 0.068   |
| dG        | 832 ± 314   | 47.9 ± 13.1 | 0.058   |
| dT        | 5.6 ± 2.1   | 0.46 ± 0.07 | 0.082   |
| Fapy-7MeG | 10³        | 0.50³       | 0.025   |
| 8-OxoG    | 14⁴         | 0.43         | 0.11    |
| Apurinic/apyrimidinic | 316³ | 0.73³ | 0.002 |

Following the assignment for the ribo-cogener (31). The spectra of compound B at room temperature contain two sets of signals (Fig. 3) that are temperature-dependent, with the coalescence in Me₆SO-d₆ at 80 °C. Each of the signal sets probably belongs to one of the isomers of compound B (Fig. 1). We assigned the isomer B₁ to be that in which the adenine ring has the hydroxyl group at position 2 and the hydrogen atom at position 3. The isomer B₂ has the opened pyrimidine ring with the carbonyl group at position 2 and the proton at position 1. The ratio of B₁ and B₂ isomers in Me₆SO-d₆ solution at 25 °C is 1:1, whereas in D₂O the ratio is 13:87.

The spectra of product C differ from those of compound B in the values of chemical shifts of the H1' and H2' protons and lack the signal at ~8.25 ppm corresponding to the proton H2. A comparison of the signals of all other non-exchangeable protons of the sugar moiety indicates that proton and carbon chemical shifts are almost identical for both the B and C products as well as the parental compound εA. This means that the sugar conformation remains very similar in all these compounds. The only differences observed are those indicating the shielding effects originated from the changes occurring within the base moiety. However, the NMR spectra of compound D are very different. The signals of the sugar moiety are absent, and only two very broad signals in the proton spectrum at ~3.5 and 6.9 ppm were observed. We have not assigned these signals and do not propose any structure for the compound.

The structures of the B and C compounds assigned by NMR were confirmed additionally by mass spectroscopy. The most abundant peaks in the B spectrum, the protonated deoxy nucleoside (m/z = 294.1) and protonated base (m/z = 178.1), differ by 18 mass units from the corresponding peaks in the εA spectrum (276.1 and 160.1, respectively). Then, the peaks in the C spectrum (266.1 and 150.1) differ by 28 units from the corresponding peaks in the B spectrum. These data are consistent with the following reaction scheme: εA + H₂O (18) → B → CO (28) → C. The presence in the mass spectrum of D peaks of m/z 316.3 and 288.3, together with the NMR data showing lack of deoxyribose in this compound, would indicate that D is a dimeric form of base moiety of compound C. The conclusion can be drawn that depurination is the final step of εA rearrangements.

Enzymatic Excision of Ethenoadenine and Its Degradation Products from DNA—Human glycosylase-ANPG-40 protein effectively excised εA from the εA:T pair in the 40-mer duplex. However, its capability to cleave εA-oligomer pretreated with NaOH decreased proportionally to the time of incubation in 0.2 N NaOH (Fig. 4A). The εA-oligomer incubated in NaOH was cleaved by E. coli formamidopyrimidine-DNA glycosylase (Fpg protein), leaving behind the β-δ-elimination product, and by endonuclease III (Nth protein), which worked by the β-elimination mechanism (Fig. 4B). At the same enzyme concentration 40-mer was cleaved to a greater extent by the Fpg than the Nth protein. When, however, both enzymes were used, only the Fpg cleavage product was found (Fig. 4B), which suggests that both enzymes recognized the same lesion, but/or Nth had a lower affinity to the lesion. Prolonged incubation of εA-oligomer in NaOH resulted also in a partial breakage of DNA at the εA site (Fig. 4, A and B). This could be because of alkali-triggered hydrolysis of abasic sites formed after non-enzymatic depurination.


The general scheme of 1,N\textsuperscript{2}-ethenoadenine rearrangements was originally proposed by Tsou and co-workers (12). Tsou and co-workers (12), employing \textsuperscript{1}H NMR and MS spectroscopy, have proven the formation of deformylated bi-imidazole nucleoside (corresponding to deoxy-cognate C, Fig. 1) from 1,N\textsuperscript{2}-ethenoadenosine. Here we present evidence, based on \textsuperscript{1}H and \textsuperscript{13}C NMR as well as on MS spectroscopy, that the first step of reaction involves formation of edA derivative hydrated at the (C(2)\textDash N(3)) bond (B1), which is in equilibrium with the pyrimidine ring-opened bi-imidazole product still retaining the formyl group (B2). In contrast to Basu et al. (13, 14), in our studies we did not observe any reversal of compound B to the parental edA during prolonged incubation of isolated B at pH 7.5. Because our studies were performed at the deoxynucleoside level, whereas the former authors (13, 14) presented evidence for this reversion at the oligodeoxynucleotide level, one cannot exclude the possibility that edA in monomer and in oligomer behaves differently.

We have additionally found that edA depurinates with a rate 20-fold higher than that of unmodified dA. Basu and co-workers (13) claimed that they did not find differences in release of edA versus A from an oligodeoxynucleotide; however, they measured depurination at pH 2 but not at neutrality. In the rearrangement pathway, we have also shown the second depurination of compound C. As judged by PAGE, under physiological conditions, about 1.5% of edA residues per week give rise to spontaneous phosphodiester bond disruption because of edA or compound D depurination (Fig. 6). This is 1–2 orders of magnitude slower than the rate of depurination of methylated bases 7MeA, 3MeA, 7MeG (32) as well as of another exocyclic base 7MeG (32) as well as of another exocyclic base (33). Nevertheless, we have shown for the first time that edA in DNA might be a source of abasic sites and spontaneous DNA strand breaks.

We have found that edA rearrangement products are not eliminated from DNA by N-methylpurine-DNA glycosylase, which participates in the repair of parental edA (Fig. 4). This conclusion is reached from the observation that the rate of alkali-induced edA \textrightarrow B conversion in oligomer (Fig. 2D) was almost the same as the rate of decrease in ANPG ability to cleave 40-mer at the edA site (Fig. 4, A and C). One of these derivatives, compound B, is excised from DNA by E. coli DNA

**Decomposition of edA in Oligomers in Physiological pH**—The edA-oligomer stored for several months in aqueous solutions at \(-30^\circ\text{C}\) was susceptible to cleavage by the Fpg and Nth proteins, suggesting that chemical rearrangement of edA also occurs spontaneously at neutrality. To determine the rate of this process, edA-40-mer was incubated up to 30 days at pH 7.4, 37 °C and digested with the Fpg and Nth proteins. Both enzymes cleaved the 40-mer in a time-dependent manner (Fig. 6). We have also observed formation of DNA breaks at the edA-site but not at other sites within an oligomer. The migration rate of the breakage product formed at neutrality was consistent with the \(\beta\)-elimination pattern. When, however, enzymatically non-digested oligomer was incubated additionally in 0.2 N NaOH for 30 min at 70 °C, the migration rate of the breakage product increased to that observed for \(\beta\)-elimination, suggesting that spontaneous breaks at the edA site in neutral pH occurred by \(\beta\)-elimination, suggesting that spontaneous breaks at the edA site in neutral pH occurred by \(\beta\)-elimination, suggesting that spontaneous breaks at the edA site in neutral pH occurred by \(\beta\)-elimination.

**DISCUSSION**

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glycosylases participating in the repair of the oxidized bases, Fpg and Nth proteins (34, 35). We surmise that the Fpg protein excises pyrimidine ring-opened isomer B2, because all known substrates of the enzyme are characterized by the presence of the carbonyl group, e.g. 8-oxoG, 4,6-diamino-5-formamidopyrimidine (FapyA), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG), its alkylated derivatives, e.g. Fapy-7MeG, and 5-hydroxycytosine (36). The A derivative is a novel substrate for the Fpg protein. The only other pyrimidine ring-opened base excised by the enzyme is ring-ruptured thymine (37). Interestingly, compound B is excised by the Fpg protein with high efficiency but only when paired with T or C but not with A or G; and thus pairs B:A or B:G might be not repaired and potentially mutagenic. The excision by Nth glycosylase of A or G; and thus pairs C:A or C:G induces a different spectrum of mutations in E. coli (40, 41). In fact, mutation for this lesion is an order of magnitude higher than for the major pyrimidine ring-opened bases, e.g. thymine glycol (17).

The other purine derivative excised by this enzyme, also with a carbonyl group, e.g. 8-oxoG, 4,6-diamino-5-formamidopyrimidine (FapyA), its alkylated derivatives, e.g. Fapy-7MeA, and 5-hydroxycytosine (36). The A derivative is a novel substrate for the Fpg protein. The only other pyrimidine ring-opened base excised by the enzyme is ring-ruptured thymine (37). Interestingly, compound B is excised by the Fpg protein with high efficiency but only when paired with T or C but not with A or G; and thus pairs B:A or B:G might be not repaired and potentially mutagenic. The excision by Nth glycosylase of A or G; and thus pairs C:A or C:G induces a different spectrum of mutations in E. coli (40, 41). In fact, mutation for this lesion is an order of magnitude higher than for the major pyrimidine ring-opened bases, e.g. thymine glycol (17).

The active center of this derivative is persistent in DNA and that some Nth glycosylases, ANPG, Fpg, and Nth, it cannot be excluded that this derivative is persistent in DNA and that some DNA-glycosylases, ANPG, Fpg, and Nth, it cannot be excluded that this derivative is persistent in DNA and that some DNA-glycosylases, ANPG, Fpg, and Nth, it cannot be excluded that this derivative is persistent in DNA and that some DNA-glycosylases, ANPG, Fpg, and Nth, it cannot be excluded.
The Pyrimidine Ring-opened Derivative of 1,N\textsuperscript{6}-Ethenoadenine Is Excised from DNA by the Escherichia coli Fpg and Nth Proteins
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