A novel activity of *E. coli* uracil DNA *N*-glycosylase Excision of isodialuric acid (5,6-dihydroxyuracil), a major product of oxidative DNA damage, from DNA

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Abstract We describe a novel activity of *E. coli* uracil DNA *N*-glycosylase (UNG) that excises isodialuric acid from DNA. Isodialuric acid is formed in DNA as a major oxidative product of cytosine. DNA substrates, which were prepared by γ-irradiation, were incubated with UNG. Following precipitation of DNA, analyses of pellets and supernatant fractions by gas chromatography/mass spectrometry showed an efficient excision of isodialuric acid from DNA by UNG. None of the other 15 identified DNA base lesions was excised. The excision of isodialuric acid indicates that the non-aromaticity of a substrate may not be a limiting factor for UNG.

Key words: Oxidative DNA damage; Hydroxyl radical; DNA repair; Modified base; Isodialuric acid

1. Introduction

Oxygen-derived species including free radicals formed in cells by endogenous and exogenous sources may cause damage to biological molecules including DNA (reviewed in [1]). Thus these species may be mutagenic and carcinogenic. Free radicals, most notably hydroxyl radical (•OH) generate a multitude of lesions in DNA and nucleoprotein (reviewed in [2,3]). DNA modifications may be repaired in cells by a variety of repair enzymes (reviewed in [4]). Free radical-induced DNA lesions are repaired by both base excision and nucleotide excision pathways, but predominantly by the former [4]. Failure of repair may lead to detrimental biological consequences such as mutations, blocking of transcription and replication.

DNA *N*-glycosylases excise modified bases from DNA by hydrolyzing glycosylic bonds between modified bases and the sugar moiety as the first step in the base excision repair pathway [4]. Of these enzymes, uracil DNA *N*-glycosylase (UNG) excises uracil, which is formed in DNA by deamination of cytosine [5]. Recently, *E. coli* UNG has been reported to excise an •OH-induced modified DNA base, 5-hydroxyuracil [6]. An oligonucleotide containing 5-hydroxy-2′-deoxyuridine was used as a substrate. In the same context, uracil was found to be excised by UNG 3–10 times more efficiently than 5-hydroxyuracil.

In the present work, we wished to investigate the substrate specificity of *E. coli* UNG toward a multitude of free radical-induced base lesions in DNA by means of the technique of gas chromatography/mass spectrometry (GC/MS). This technique permits the identification and quantification of a large number of pyrimidine- and purine-derived base lesions in the same DNA sample [7]. Thus, the substrate specificity of a particular DNA *N*-glycosylase or any other repair enzyme toward numerous DNA base lesions can be studied simultaneously under the same conditions as we have demonstrated recently in the case of *E. coli* Fpg protein and endonuclease III [8,9].

2. Materials and Methods

2.1. Materials

Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Modified DNA bases, their stable isotope-labeled analogues, and materials for GC/MS were obtained as described [7]. *E. coli* uracil DNA *N*-glycosylase was purchased from Boehringer-Mannheim. Calf thymus DNA was purchased from Sigma Chemical Co. Dialysis membranes with a molecular weight cutoff of 6000–8000 were obtained from Fisher Scientific Co.

2.2. Irradiations

Calf thymus DNA was dissolved in 10 mM phosphate buffer (pH 7.4) (0.3 mg/ml) and then dialyzed against 10 mM phosphate buffer. Aliquots of the DNA solution were bubbled with air or oxygen-free argon for 30 min and subsequently irradiated with γ-rays in a ^{60}Co γ-source at a dose of 50 Gy (dose rate 65 Gy/min). Following irradiation, samples were dialyzed extensively against 10 mM phosphate buffer (pH 7.4).

2.3. Enzymatic assays

Aliquots of DNA samples containing 50 μg of DNA were dried in a SpeedVac under vacuum at room temperature. They were dissolved in 100 μl of 50 mM phosphate buffer (pH 8) containing 1 mM EDTA, 1 mM dithiothreitol and bovine serum albumin (0.1 mg/ml). Aliquots of active or inactivated UNG (10 units (U)) were added to each sample (the manufacturer of UNG defines 1 U as the amount of UNG necessary to completely degrade 1 μg of uracil-containing single-stranded

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Abbreviations: UNG, uracil DNA *N*-glycosylase; •OH, hydroxyl radical; GC/MS-SIM, gas chromatography-mass spectrometry with selected-ion monitoring; U, unit; 5-OH-5-MeHyd, 5-hydroxy-5-methylhydantoin; 5-OH-Hyd, 5-hydroxyhydantoin; 5-OHMeUra, 5-(hydroxymethyl)uracil; 5-OH-Ura, 5-hydroxyuracil; 5-OH-Cyt, 5-hydroxycytosine; Thy glycol, thymine glycol; 5,6-diOH-Ura, 5,6-dihydroxyuracil (isodialuric acid); FapyAde, 4,6-diamino-5-formamidopyrimidine; 8-OH-Ade, 8-hydroxyadenine; 2-OH-Ade, 2-hydroxyadenine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH- Gua, 8-hydroxyguanine; 5,6-diHThy, 5,6-dihydrothymine; 5,6- diHUra, 5,6-dihydrouracil; 5-OH-6-HThy, 5-hydroxy-6-hydrothymine; 5-OH-6-HUra, 5-hydroxy-6-hydrouracil.

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DNA at 37°C in 60 min). This was followed by incubation at 37°C for 15 min, 30 min, 1 h or 2 h. Some DNA samples were treated in the same manner but without addition of UNG. Inactivation of UNG was done by heat treatment at 160°C for 1 h. After incubation of DNA samples, 250 μl of cold ethanol (−20°C) was added to each sample, and the resulting mixture was kept at −20°C for 2 h. The mixture was centrifuged at 4°C for 30 min at 10,000 rpm. The precipitated DNA pellets and the supernatant fractions were separated, and subsequently dried in a SpeedVac under vacuum at room temperature. The dependence of the excised amount of the substrate on the enzyme amount was determined using 2.5 U, 5 U, 7 U and 10 U of UNG, and 50 μg of irradiated DNA in 100 μl of buffer A. Incubation time was 1 h. Treatment of samples after incubation was as described above. For determination of the excised amount of the substrate as a function of the substrate concentration, 12.5 μg, 25 μg, 50 μg, and 75 μg of irradiated DNA were supplemented with 87.5 μg, 75 μg, 50 μg and 25 μg of unirradiated DNA, respectively. An additional sample containing 100 μg of irradiated DNA was also used. Three replicates of these samples were incubated in 200 μl of buffer A with active UNG (10 U) or without UNG for 1 h at 37°C. Subsequently, the samples were treated as described above.

2.4. Gas chromatography/mass spectrometry

The concentration of DNA in pellets was determined by the absorbance at 260 nm (absorbance of 1 = 50 μg of DNA/ml). The recovery of DNA by precipitation with ethanol was almost 100%. Aliquots of stable isotope-labeled analogues of modified DNA bases were added as internal standards [7] to DNA pellets and to supernatant fractions. Samples were then lyophilized. Dried pellets were hydrolyzed and derivatized as described [10]. Supernatant fractions were derivatized with no prior hydrolysis. Analyses of derivatized samples for modified DNA bases were performed by GC/MS with selected-ion monitoring (SIM) [10].

3. Results

This study was undertaken to investigate the ability of *E. coli* UNG to excise DNA base lesions that are produced in DNA by reactions of free radicals. For this purpose, we used DNA substrates that were prepared by exposure of DNA in dilute aqueous solution to ionizing radiation under oxic or anoxic conditions. Recent studies have shown that the types of DNA base lesions and their yields in irradiated DNA depend considerably on oxygen [11,12]. Modified DNA bases that were identified in DNA samples in this work are given in Table 1. Uracil derivatives are produced from the cytosine moiety [2,3], and may exist in DNA as their cytosine-derived analogues prior to analysis.

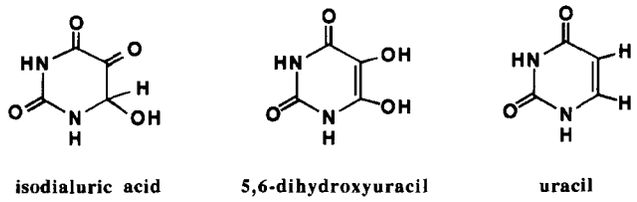

Fig. 1. The structures of isodialuric acid, 5,6-dihydroxy-uracil and uracil.

The results obtained showed that, of the DNA base lesions identified in this work, only 5,6-dihydroxyuracil (5,6-diOH-Ura) was removed from DNA by active *E. coli* UNG. 5,6-Dihydroxyuracil is the enol form of isodialuric acid (6-hydroxy-2,4,5-[1*H*,3*H*,6*H*]-pyrimidinetrion e), which is the prevalent form in aqueous solution or in the crystalline state [13]. Isodialuric acid is converted into its enol form during derivatization, and thus it is detected as 5,6-dihydroxyuracil by GC/MS [14]. A comparison of the structures of isodialuric acid, 5,6-dihydroxyuracil and uracil is shown in Fig. 1. In this work, 5,6-dihydroxyuracil was detected in DNA pellets as well as in the supernatant fractions. Its cytosine-derived analogue 5,6-dihydroxycytosine was not observed. It should be pointed out that the enolization of dialuric acid (5-hydroxy-2,4,6-[1*H*,3*H*,5*H*]-pyrimidinetrion e) may also yield 5,6-dihydroxyuracil. However, dialuric acid is spontaneously oxidized in aqueous solution and yields alloxan [13]. Alloxan is converted into 5-hydroxyhydantoin upon acidic treatment [7]. Alloxan is a substrate for endonuclease III [9].

The amount of isodialuric acid in γ-irradiated DNA is shown in Fig. 2. When DNA was γ-irradiated under oxic conditions, an ≈8-fold increase in the amount of this compound over the background level was observed at the radiation dose (50 Gy)

Table 1
Base products and their yields (nmol/mg of DNA[a]) in DNA

Product	Control DNA	γ-Irradiated DNA	
		Under air	Under argon
5-OH-5-MeHyd	0.070 ± 0.005	0.625 ± 0.107	0.102 ± 0.004
5-OH-Hyd	0.138 ± 0.026	0.412 ± 0.020	0.154 ± 0.016
5-OHMeUra	0.021 ± 0.004	0.055 ± 0.009	0.073 ± 0.004
5-OH-Ura	0.077 ± 0.007	0.393 ± 0.011	0.114 ± 0.006
5-OH-Cyt	0.100 ± 0.018	0.623 ± 0.039	0.195 ± 0.021
Thy glycol	0.164 ± 0.017	1.61 ± 0.09	0.348 ± 0.027
5,6-diOH-Ura	0.036 ± 0.007	0.302 ± 0.015	0.060 ± 0.009
FapyAde	0.117 ± 0.007	1.40 ± 0.079	1.75 ± 0.09
8-OH-Ade	0.233 ± 0.003	0.876 ± 0.027	0.514 ± 0.082
2-OH-Ade	0.032 ± 0.003	0.053 ± 0.005	0.029 ± 0.011
FapyGua	0.051 ± 0.003	1.20 ± 0.021	1.64 ± 0.08
8-OH-Gua	0.445 ± 0.071	2.34 ± 0.07	1.05 ± 0.085
5,6-diHThy	n.d.[b]	n.d.	1.11 ± 0.037
5,6-diHUra	n.d.	n.d.	0.669 ± 0.061
5-OH-6-HThy	n.d.	n.d.	0.505 ± 0.028
5-OH-6-HUra	n.d.	n.d.	0.105 ± 0.006

[a]Values represent the mean ± standard deviation from three independent measurements.
[b]Not detected.

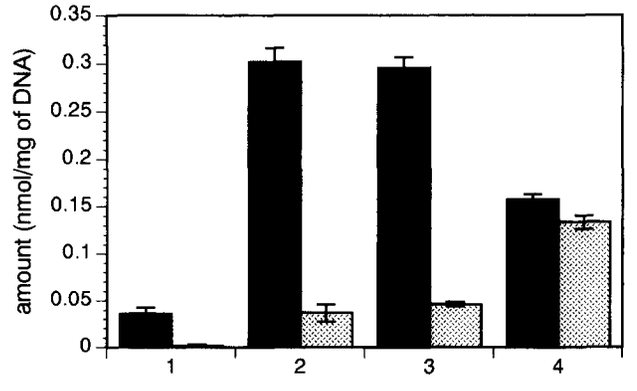

Fig. 2. Amounts of isodialuric acid in pellets and supernatant fractions of DNA samples γ-irradiated under oxic conditions. Treatment: (1) control DNA (not irradiated); (2) irradiated DNA after incubation without the enzyme for 1 h; (3) irradiated DNA after incubation with 10 U of inactivated UNG for 1 h; (4) irradiated DNA after incubation with 10 U of active UNG for 1 h. Dark columns = pellets; light columns = supernatant fractions. Graphs represent the mean ± S.D. from three independent experiments.

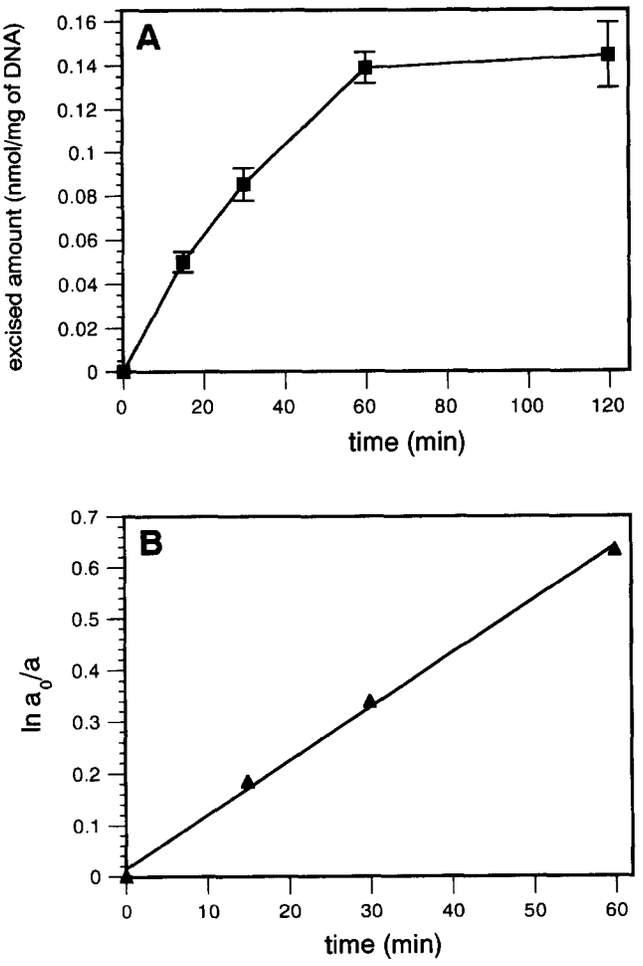

Fig. 3. (A) The time course of the excision of isodialuric acid by active UNG (10 U). The measurement points represent the mean ± S.D. from three independent experiments. DNA samples irradiated under oxic conditions were used for this experiment. (B) The plot of the logarithm of the ratio of the initial amount (a_0) to the remaining amount (a) of isodialuric acid in DNA as a function of the incubation time.

used (Table 1). Its yield was much lower in DNA samples irradiated under anoxic conditions (Table 1). DNA samples irradiated under oxic conditions were used throughout this work for studies of UNG-mediated excision of isodialuric acid. The amount of isodialuric acid excised from DNA by active UNG corresponded to its amount found in the supernatant fractions (Fig. 2). Small but detectable levels of isodialuric acid were also found in the supernatant fractions of the samples incubated without the enzyme or with inactivated enzyme. However, the amounts were substantially smaller than that found in the supernatant fractions of the samples incubated with the active enzyme.

The excision of isodialuric acid from DNA by UNG has characteristics of a Michaelis-Menten reaction (Fig. 3A). UNG-mediated release of isodialuric acid into the supernatant fraction progressed up to 1 h of incubation with no additional excision observed up to 2 h. A plot of the logarithm of the ratio of the initial amount (a_0) to the remaining amount (a) of isodialuric acid in DNA as a function of the incubation time shows that the excision follows first-order kinetics up to 60 min of incubation (Fig. 3B). The slop of this plot yielded a rate constant of 0.011 min^{-1} (rate constant (k) = $\ln(a_0/a)$/time] and a half-life (τ) of 63 min ($\tau = 0.693/k$). UNG-mediated excision of isodialuric acid was also determined as a function of the substrate concentration. Fig. 4 shows a Lineweaver-Burk plot of the data obtained. This plot indicated an apparent K_m of ≈134 nM and an apparent V_{max} of ≈0.74 nM/min.

4. Discussion

The results of this work provide evidence for a novel activity of *E. coli* UNG that excises isodialuric acid from DNA as an *N*-glycosylase. Fifteen other modified bases, which are produced in DNA along with isodialuric acid, are not substrates for UNG. Previous work has shown that isodialuric acid was not a substrate for *N*-glycosylases such as *E. coli* Fpg protein or endonuclease III, although a majority of the DNA base lesions were substrates for these enzymes [8,9]. Isodialuric acid is a product of cytosine, which is formed in DNA by reactions of •OH (reviewed in [2,3,15]) or by non-radical pathways upon exposure of DNA to oxidizing agents [16,17]. As our results indicate, the yield of this product is comparable to the yields of other major products (see section 3). Recently, the formation of isodialuric acid has been shown to occur in genomic DNA of cultured mammalian cells (reviewed in [3]) and in genomic DNA of animals in vivo [18–20] that were exposed to agents causing oxidative DNA damage. The yield of isodialuric acid was comparable to those of the other DNA base products. Moreover, chromatin samples isolated from human cancerous tumors have been found to contain this compound in greater amounts than those isolated from surrounding normal tissues [21,22].

The finding that isodialuric acid is a substrate for *E. coli* UNG brings the number of reported non-uracil substrates to three, including 5-fluorouracil [23] and 5-hydroxyuracil [6]. However, we were unable to detect the release of 5-hydroxyuracil from substrates containing substantial amounts of this lesion. This discrepancy may have several explanations. Hatahet et al. [6] used an oligonucleotide containing 5-hydroxyuracil as the exclusive lesion, whereas we used DNA containing numer-

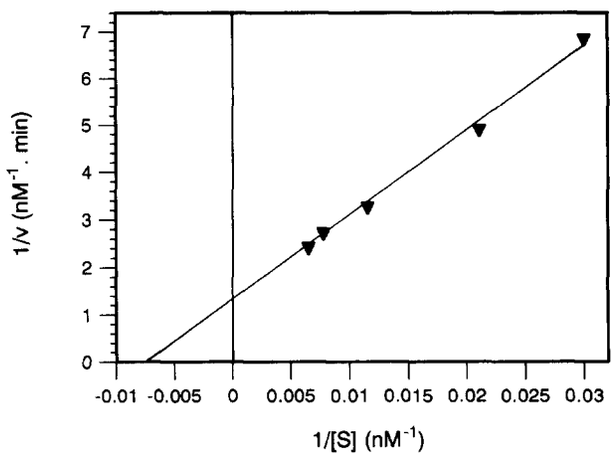

Fig. 4. The Lineweaver-Burk plot of the excision. v = initial reaction rate; [P] = concentration of isodialuric acid. DNA samples irradiated under oxic conditions were used for this experiment.

ous lesions. There was also a moderate difference in the enzyme:substrate ratio used in their study (1 ng UNG/fmol 5-hydroxyuracil) compared to our study (0.17 ng UNG/fmol 5-hydroxyuracil). Furthermore, 5-hydroxyuracil may not exist per se in DNA, but instead it may be converted from two known •OH-induced precursors cytosine glycol or uracil glycol [2,3]. Deamination and/or dehydration of cytosine glycol or uracil glycol yield 5-hydroxyuracil upon acidic treatment [9], but it is unknown at present whether or not such a conversion takes place spontaneously in DNA prior to hydrolysis or derivatization. In addition, no kinetics parameters were reported for 5-hydroxy-uracil in the paper by Hatahet et al. [6], so it is difficult to estimate the relative efficiency of its putative removal from DNA by UNG compared to removal of uracil. The K_m for 5-fluorouracil was reported to be about 5 μM, and was about 18 times higher than that of uracil in the same study [23]. Our finding that the K_m for isodialuric acid is 134 nM compares to the reported K_m for uracil of 40 nM [5].

The recent availability of the X-ray crystal structure for *E. coli* UNG allows some rationalization of its recognition of isodialuric acid [24]. The ligand binding pocket of UNG makes a number of specific contacts with uracil that exclude binding of the normal DNA because due to steric clashes with the ring of Tyr 90 or unfavorable repulsive interactions with polar groups lining the pocket. In addition, specific hydrogen bonds between the N3, O4, O2, and N1 of uracil and adjacent amino acid residues must also takes place. Isodialuric acid must somehow meet these ligand binding requirements despite its non-aromatic (non-planar) character. Modelling of isodialuric acid into the pocket indicates that it should be able to meet the hydrogen bonding requirements of the ligand binding pocket. In addition, the C5 carbonyl oxygen should present much less of a steric clash potential with Tyr-90 compared to the C5 methyl group of thymine. The remaining C6 hydroxyl group may counterbalance potential steric clashes or suboptimal positioning of groups for hydrogen bonding by forming additional stabilizing hydrogen bonds not possible with uracil.

In conclusion, this work provides evidence for a novel activity of *E. coli* UNG. Thus, as suggested previously [6], UNG may also be an important component in the repertoire of cellular proteins that repair frequently-occurring oxidative DNA damage which includes isodialuric acid. Although the potential biological effects of isodialuric acid are unknown, its efficient removal from DNA by UNG suggests that it may have potentially toxic and/or mutagenic effects on the gene expression machinery. It will be important in future studies to address the DNA base-pairing properties of isodialuric acid as well as the structural basis for its structural recognition and excision by UNG.

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