Partial Purification and Characterization of a Uracil-DNA Glycosylase from Wheat Germ*

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A uracil-DNA glycosylase has been purified over 1,000-fold from wheat germ, the first such repair activity isolated from a higher plant. The enzyme has a molecular weight of approximately 27,000 and is resistant to metal ion chelators, but inhibited by high concentrations of either mono or divalent cations. This glycosylase is able to release uracil from the mono-nucleotides dUMP and dUTP or from wheat germ RNA.

Twelve pyrimidine analogues which closely mimic uracil and deoxyuridine and the nucleoside uridine were examined for their ability to inhibit glycosylase activity. However, only 5-azauracil and 6-aminouracil inhibited enzymatic release of uracil to the same degree as uracil itself. An inhibitor induced by bacteriophage T5 which inhibits Escherichia coli uracil-DNA glycosylase has been shown not to affect the glycosylase isolated from wheat germ, indicating that these two enzymes differ. The ability of the wheat germ uracil-DNA glycosylase to completely remove available uracil from synthetic DNA substrates in which thymine had been replaced by uracil in varying percentages was also examined and found not to depend on percentage of uracil in the substrates.

Uracil is not a usual constituent of DNA in either eucaryotes or procaroytes. However, uracil can be introduced as a nascent DNA if intracellular dUTP concentrations are abnormally high, allowing incorporation of this nucleotide into the growing chain by DNA polymerase (1). Uracil can also arise in mature DNA if cytosine bases are deaminated, either spontaneously (2) or by chemical mutagens such as bisulfite (3). Uracil incorporation results in Ade-Ura base pairs of approximately the same size and shape as Ade-Thy pairs, but cytosine deamination replaces Gua-Cyt with Gua-Ura pairings leading to transition mutations if uncorrected prior to the next round of replication.

Uracil-DNA glycosylases remove uracil where it occurs in DNA by hydrolytically cleaving the N-glycosyl bond between the base and sugar, leaving an apyrimidinic site which is subsequently recognized and repaired by other enzymes (4, 5). Uracil-DNA glycosylases have been purified from several bacterial and eucaryotic organisms including Escherichia coli (6), Bacillus subtilis (7), and human blast cells (8) and found to be generally similar in such physical and enzymatic parameters as molecular weight, substrate specificity, and lack of metal cofactor requirements. So far, no comparable repair activity has been purified and characterized in higher plant tissues.

This report details the characterization of one such uracil-DNA glycosylase, purified over 1000-fold from wheat germ. The plant enzyme has been found to resemble already characterized uracil-DNA glycosylases in most respects, yet differs from the E. coli enzyme in its response to the uracil-DNA glycosylase inhibitor induced in phage T5-infected bacteria (9), indicating that some variation exists between these two examples of eucaryotic and procaroytic DNA repair enzymes. In addition, the ability of the wheat germ glycosylase to remove uracil from synthetic DNA substrates was examined as a function of percentage of uracil present in the DNA.

EXPERIMENTAL PROCEDURES

Materials—Wheat germ (W-0125, untreated, type II) was purchased from Sigma and stored at 0–4 °C prior to use. Also obtained from Sigma were the uracil analogues orotic acid, 5-fluorouracil, 5-bromouracil, 5-aminouracil, 6-aminouracil, 2-thiouracil, 6-azauracil, and 6-methyluracil. All protein standards except chemotryptsinogen (Pharmacia Chemicals) were purchased from Sigma.

Ultrapure ammonium sulfate came from Schwarz/Mann, 8-hydroxyquinoline from Eastman Organic Chemicals, and 5-azauracil from ICN Pharmaceuticals. Bio-Gel HTP hydroxylapatite, Bio-Gel A-1.5m, and the anion exchange resin AG 1-X8 (formate form) were purchased from Bio-Rad; DEAE-cellulose, F-11 phosphocellulose, and No. 1 chromatography paper from Whatman; and Sephadex G-100 from Pharmacia Chemicals.

[6-3H]Uridine was obtained from New England Nuclear, [6-3H]dUTP came from Moravek Biochemicals, and [6-3H]dUMP from Schwarz/Mann.

Preparation of DNA Substrates—Phage T5 DNA labeled with [6-3H]Uridine was prepared as described by Warner et al. (10) using E. coli strain BD1157 (mut. ung) and a T5 strain. The T5 DNA contained approximately 4.25% uracil as a percentage of all its bases. Thus, there are about 22.5 bases between uracils. [5-3H]dUTP-containing DNA was made from nick-translated calf thymus DNA according to Krokan and Wittwer’s procedure (11). Within the nick-translated regions of the calf thymus substrate, uracil makes up approximately 28% of the bases so there are about 2.6 bases separating uracils.

The poly(dA-dT) substrates were synthesized in a reaction mixture with a total volume of 1500 μl containing 10 μl of 2.8 mM poly(dA-dT) purchased from Calbiochem-Behring, 500 μl of 0.2 M KPO4, pH 7.5, 10 μl of 1 M MgCl2, 15 μl of 3.1 mM dATP (Sigma), 2 μl of 1.0 mM mercaptoethanol, 40 μl of 67 mM [5-3H]dUTP with a specific activity of 15 Ci/mmol (Moravek Biochemicals), 1 μl of E. coli DNA polymerase I (6000 units/ml) from Bethesda Research Laboratory, and varying ratios of unlabeled dUTP (Sigma) and dTTP (Sigma) such that there was a total of 50 nmol of the two nucleotides present in the reaction mixture. The ratio of these two nucleotides was varied to give DNA substrates with ratios of uracil to thymine of 100:0, 50:50, or 5:5 in their newly synthesized regions. All unlabeled nucleoside triphosphates were examined for purity prior to use by thin layer chromatography on Polygram Cel 300 polyethyleneimine plates (Merck/Nagel and Co., distributed by Brinkmann Instruments, Inc.) and found to be essentially uncontaminated with mono- or diphos-
phates. Reactions were typically incubated at 37 °C for about 1½ h (longer reaction periods resulted in loss of radioactivity from polynucleotide) followed by addition of 200 µl of 0.1 M EDTA to halt polymerase activity. These preparations were dialyzed against 0.01 M Tris-HCl, pH 7.5, 0.2 M EDTA overnight to remove unincorporated nucleotides and purified on a Bio-Gel A-1.5m column to remove small oligonucleotides. For the poly(dA-dT) nucleic acids, where the dA: dT ratio was 100:0, there was an average of one base between uracils in the newly synthesized region; where the dU: dT ratio was 50:50, there was an average of three bases between uracils; and where the ratio was 5:98, there was an average of 39 bases between uracils.

Uracil-DNA Glycosylase Assay Procedure—The assay for glycosylase activity is the same as that described in Duncan et al. and Warner and Rockstroh (12, 13) using columns (0.3 x 15 cm) containing Bio-Rad AG 1-X8 formate packed to a height of 3 cm. Unless otherwise specified, the standard incubation conditions were 30 min at 37 °C in an assay mixture containing 30 µl of 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 10 µl of 0.04 M EDTA, pH 7.8, 10 µl of 0.1 M diethiothreitol, 10 µl of 1 mg/ml of bovine serum albumin, and varying amounts of T5 DNA-uracil containing uracil in a final assay volume of 200-210 µl. Where indicated, the T5 DNA substrate was replaced with uracil-containing calf thymus DNA or poly(dA-dT). All reactions were initiated with the addition of enzyme and stopped with 500 µl of 0.1 M ammonium formate, pH 4.2. After addition of formate, the reaction mixture was cooled on ice and then applied to the columns. Counting was performed in Aquasol 2 (New England Nuclear) containing counting mixture in a Beckman LS 7800 liquid scintillation spectrometer. One unit of uracil-DNA glycosylase is defined as the amount of enzyme needed to release 1 nmol of uracil from phage T5 DNA substrate/min under standard conditions. Typically, 0.001-0.002 unit of enzyme was used/assay during the characterization.

Molecular Weight—Centrifugation through linear 10-25% glycerol gradients was done using glycerol solutions containing 0.01 M potassium phosphate, pH 7.5, and 1 mM diethiothreitol. The samples were centrifuged for 42-43 h in an SW 50.1 head at 46,000 rpm in a Beckman L5-50 ultracentrifuge maintained at 2-4 °C. 150-µl fractions were collected and assayed for the presence of cytochrome c (A550), ovalbumin (A280), bovine serum albumin (A280), and uracil-DNA glycosylase.

Gel filtration was performed in a column (1 x 25 cm) equilibrated prior to use with 0.01 M potassium phosphate, pH 7.5, 1 mM diethiothreitol, and 15% glycerol. Bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome c protein standards were used to calibrate the column. The flow rate was maintained near 2.5 ml/h.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis to determine the uracil-DNA glycosylase molecular weight was done on 15% acrylamide gels according to the procedure of Laemmli and Favre (14). Protein staining was accomplished using the silver-staining technique described by Merril et al. (15). Wheat germ uracil DNA glycosylase was prepared for electrophoresis by purifying the enzyme through the DEAE-cellulose stage as detailed under "Results." This preparation was loaded onto a Beckman LS 7800 liquid scintillation spectrometer. One unit of uracil-DNA glycosylase is defined as the amount of enzyme needed to release 1 nmol of uracil from phage T5 DNA substrate/min under standard conditions. Typically, 0.001-0.002 unit of enzyme was used/assay during the characterization.

Miscellaneous—Partially purified phage T5 uracil-DNA glycosylase inhibitor and E. coli uracil-DNA glycosylase were generous gifts of Richard Leder (University of Minnesota). Inhibitor was always added to assay mixtures before the enzyme.

[6-3H]UMP-labeled wheat germ RNA was generously donated by James Skuzeski (University of Minnesota).

Base analysis of the phage T5 DNA substrate and reaction products from glycosylase assays was carried out using descending paper chromatography for about 18 h as described by Warner et al. (10). The yields of cytosine and uracil from the product DNA were determined by comparing the chromatographic bands with known standards.

Radioactivity was measured using the nicked DNA assay described by Kane and Linn (18) using unincorporated and acid-depurinated PM2 [3H]DNA.

RESULTS

Purification

Preparation of Crude Extract—All procedures were carried out at 0-4 °C unless otherwise stated. 500 g of wheat germ were suspended in 2 liters of buffer containing 0.01 M potassium phosphate, pH 7.3, 15% glycerol, and 1 mM dithiothreitol (PGD buffer) and homogenized for 1 min at high speed in a Waring blender. Cell wall debris was removed by centrifugation for 15 min at 10,000 x g. This supernatant was then filtered through several layers of cheesecloth. 150 ml of 5% (w/v) streptomycin sulfate were slowly added to the approximately 2 liters of turbid supernatant with gentle stirring and allowed to sit for 20 min on ice followed by centrifugation for 15 min at 10,000 x g to remove nucleic acids.

Ammonium Sulfate Precipitation—Following precipitation of the nucleic acids with streptomycin, the supernatant was brought to 50% saturation with solid ammonium sulfate while maintaining the pH at 7.3 and then allowed to sit on ice for 30 min with stirring. This suspension was centrifuged for 15 min at 15,000 x g. The 50-80% fraction was found to contain most uracil-DNA glycosylase activity and was redissolved in 750 ml of PGD buffer and dialyzed against this buffer overnight to remove ammonium sulfate.

DEAE-cellulose Chromatography—The diazyl redissolved pellet was divided into 250-ml portions for easier handling. Each portion was loaded onto a DEAE-cellulose column (8 x 30 cm) equilibrated with PGD buffer. The column was then washed with PGD. Uracil-DNA glycosylase activity eluted prior to the bulk of wheat germ protein without requiring a salt gradient. Peak fractions were collected, pooled, and dialyzed against PGD buffer.

Phosphocellulose Chromatography—The combined fractions containing uracil-DNA glycosylase activity were next loaded onto a phosphocellulose column (2.7 x 30 cm) equilibrated with PGD buffer. The column was washed with PGD buffer and then eluted with a linear 0.01-0.4 M KCl gradient. Fractions containing peak activity were pooled, and the protein was precipitated with a saturating concentration of solid ammonium sulfate followed by centrifugation at 15,000 x g for 15 min. The pellet was resuspended in 20 ml of PGD buffer and dialyzed against this buffer to remove ammonium sulfate.

Sephadex G-100-Chromatography—This solution was loaded onto a Sephadex G-100 column (27 x 30 cm) equilibrated with PGD buffer and eluted with the same buffer. Fractions containing uracil DNA-glycosylase activity were pooled.

Bio-Gel Hydroxyapatite Chromatography—The pooled uracil-DNA glycosylase fractions were chromatographed on a hydroxyapatite column (1.0 x 25.0 cm) equilibrated with PGD buffer. To elute enzyme activity, a linear 0.01-0.4 M potassium phosphate gradient was used. Peak glycosylase fractions were pooled, and the protein was precipitated with a saturating concentration of solid ammonium sulfate followed by centrifugation. The pellet was resuspended and dialyzed against PGD buffer.

Bio-Gel A-1.5m Chromatography—The concentrated glycosylase sample was applied to a Bio-Gel A-1.5m column (1.8 x 19 cm) and eluted with PGD buffer. The pooled glycosylase fractions were then concentrated via Millipore filtration.

Second Phosphocellulose Chromatography—The concen-
The pooled enzyme fractions showed little loss of activity when stored at −20 °C in 40% glycerol. The purification of the enzyme is summarized in Table I.

The possible presence of double- and single-stranded DNA exonucleases as well as nonspecific endonucleases was examined in the final enzyme preparation. These nucleases were found to be less than 0.2% of the uracil-DNA glycosylase activity for double-stranded exonuclease, less than 2.0% for single-stranded exonuclease, and less than 0.1% for nonspecific endonucleases. An apurinic endonuclease co-chromatographed with the wheat germ uracil-DNA glycosylase activity during initial stages of purification, but had resolved away from peak glycosylase activity by the second phosphocellulose column. Apurinic endonuclease activity was less than 0.1% of glycosylase activity after the final stage of purification.

**Verification of Uracil Release**

To determine which pyrimidine bases were released by the partially purified glycosylase activity, the enzyme was incubated with uracil-containing phage T5 DNA long enough to release virtually all of the available uracil. This substrate contains approximately 40% of its tritium in cytosine, 50% in thymine, and the remaining 10% in uracil, making it particularly sensitive to the release of either cytosine or thymine. Fig. 1 shows that all of the radioactivity migrates as a single peak exactly paralleling the mobility of the uracil standard. No radioactivity co-migrates with either cytosine or thymine standards on the chromatograph, demonstrating that the wheat germ glycosylase liberates exclusively uracil from among the three pyrimidines potentially available in the DNA substrate.

**Table I**

| Step             | Volume | Protein concentration | Specific activity | Purification |
|------------------|--------|-----------------------|-------------------|--------------|
|                  | ml     | mg/ml                 | units/mg protein  |              |
| Crude extract    | 2000   | 14                    | 61.6              | 0.0022       | 1            |
| DEAE-cellulose   | 560    | 1                     | 17.4              | 0.031        | 14           |
| Phosphocellulose | 210    | 0.28                  | 12.5              | 0.23         | 104          |
| Sephadex G-100   | 75     | 0.33                  | 5.9               | 0.24         | 105          |
| Hydroxylapatite | 30     | 0.33                  | 5.4               | 0.55         | 250          |
| Bio-Gel A-1.5m   | 15     | 0.26                  | 2.6               | 0.68         | 310          |
| Phosphocellulose | 14     | 0.05                  | 2.45              | 3.5          | 1590         |

**Physical Characterization of the Enzyme**

**Molecular Weight**—The molecular weight of the wheat germ uracil-DNA glycosylase was determined to be 27,000 by comparison with the elution position of four standards of known molecular weight on a Sephadex G-100 column (Fig. 2).

Ultracentrifugation on glycerol gradients was also used to obtain a molecular weight by relating the sedimentation behavior of three standards with known $s_{20w}$ values to that of wheat germ glycosylase (data not shown). If the partial specific volume of the glycosylase is assumed to be close to 0.725, as is true for many proteins, the molecular weight was calculated to be about 28,000 (19).

By correlating glycosylase activity present in fractions collected from a hydroxylapatite column with band intensity when these same fractions are examined on a sodium dodecyl sulfate-polyacrylamide gel, it is possible to match a particular band with the wheat germ glycosylase activity. As Fig. 3 shows, the band correlating best with glycosylase activity (indicated by the arrow) has a molecular weight of approxi-

![FIG. 2. Molecular weight determination by gel filtration. Gel filtration was performed on a Sephadex G-100 column using the four protein standards, bovine serum albumin (BSA) $(M, = 67,000)$, ovalbumin $(M, = 45,000)$, chymotrypsinogen $(M, = 25,000)$, and cytochrome c $(M, = 12,500)$.](image)

![FIG. 3. Molecular weight determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of active fractions from hydroxylapatite chromatography. The outer lanes contain molecular weight standards (MW Std). The numbers arranged vertically along the left side of the gel correspond to the following standards: 1, hemoglobin subunit $(M, = 16,000)$; 2, soybean trypsin inhibitor $(M, = 21,000)$; 3, carbonic anhydrase $(M, = 29,000)$; 4, malate dehydrogenase $(M, = 34,000)$; and 5, aldolase $(M, = 40,000)$. Fraction numbers are arranged along the bottom of the gel, and counts/min in uracil released by a given fraction are directly above that fraction's lane. The protein band showing correspondence with uracil release is indicated by the arrow to the right.](image)
mately 25,000, which agrees with the sizes obtained by gel filtration and ultracentrifugation for this enzyme. Assuming the designated band does represent the glycosylase, Fig. 3 also shows that the activity is a relatively minor component among the plant's complement of proteins.

**Temperature and pH Optima**—The wheat germ glycosylase is moderately stable at elevated temperatures, retaining about 50% of full activity after 1½ h of incubation at 45 °C. One minute at 60 °C abolishes all activity.

The pH profile (Fig. 4) shows measurable activity between 6.5 and 8.5, indicating the enzyme has a broad tolerance of varying pH conditions. This profile also shows that at a given pH, the enzyme exhibits greatest activity in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered medium.

**Effects of Metal Ions and Metal Ion Chelators**—The wheat germ uracil-DNA glycosylase is strongly inhibited by high concentrations of monovalent cations (Fig. 5A). The inhibition patterns seen when the glycosylase is exposed to K⁺, Na⁺, and Li⁺ are alike, indicating that inhibition is due to increased ionic strength in the reaction medium rather than the specific effects of a particular ionic species upon enzyme activity. The patterns for the two divalent species, Mg²⁺ and Ca²⁺ (Fig. 5B), are also similar, again showing that ionic strength is the important factor inhibiting glycosylase activity.

Fig. 5C presents the effects of the ionic chelator EDTA on glycosylase activity. The chelator does not appear to inhibit activity at concentrations less than 20 mM. However, at 50 mM, EDTA shows significant inhibition, but this effect is amplified by addition of MgCl₂, indicating that the ionic strength of EDTA rather than its chelating ability is the important factor inhibiting enzyme activity. Furthermore, no requirement for metal ions was observed during purification and storage. The nonionic chelator 8-hydroxyquinoline was also tested on the wheat germ uracil-DNA glycosylase at concentrations up to its solubility limit in an aqueous buffer (about 3 mM) and found to have no inhibitory effect upon enzymatic activity.

N-ethylmaleimide, a sulfhydryl group-blocking agent, was tested on the glycosylase and only reduced activity to 75% of control levels at 50 mM, showing that sulfhydryl groups probably do not play an important part at the active site of the glycosylase.

**Uracil Analogues and Glycosylase Inhibitors**

**Uracil Analogues**—Fig. 6 shows the inhibitory effects of 14 nucleoside and pyrimidine analogues closely resembling uracil structurally. Wheat germ glycosylase was inhibited by uracil, 6-aminouracil, or 5-azauracil, while pyrimidines as similar to uracil as 6-azauracil, 5-fluorouracil, and 5-aminouracil do not appear to markedly decrease glycosylase activity below control levels. Thymine and cytosine, the two pyrimidines normally found in DNA, also do not inhibit activity. All compounds were present at 1 mM.

**Effects of T5-induced Uracil-DNA Glycosylase Inhibitor**—When bacteriophage T5 infects E. coli, it induces an inhibitor of host uracil-DNA glycosylase activity (9), presumably to somehow expedite the infection process. In an attempt to determine whether this inhibitor had similar effects on wheat germ glycosylase, the action of the uracil glycosylase inhibitor induced by phage T5 was compared on both wheat germ and E. coli glycosylases. Fig. 7 shows clearly that amounts of inhibitor which drastically diminish the activity of E. coli glycosylase have no apparent effect on the plant enzyme. Because the substrate is the same in both cases, this is evidence for a structural difference between the E. coli and wheat germ enzymes allowing inhibitor-enzyme interaction with the bacterial glycosylase, but preventing it with the plant enzyme.

**Substrate Specificity**

**Ability to Release Uracil from dUMP and dUTP**—To explore the ability of the wheat germ enzyme to release uracil from mononucleotides as well as polynucleotides, uracil release from [6-³H]dUMP and [5-³H]dUTP by the glycosylase was measured. Under conditions such that over 50% of available uracil from the T5 DNA substrate was liberated, no release of free uracil from either mononucleotide was detected.
Wheat Germ Uracil Glycosylase

Fig. 7. Effects of T5-induced inhibitor. The T5-induced E. coli uracil-DNA glycosylase inhibitor was incubated with both plant and bacterial enzymes under the same standard assay conditions. □—□, wheat germ glycosylase activity; ○—○, E. coli glycosylase activity.

Table II

| Species containing [3H]uracil | Other additions | Available [3H] uracil released |
|------------------------------|-----------------|-------------------------------|
| DNA                          |                 | 52                            |
| dUMP                        |                 | 0                             |
| dUTP                        |                 | 0                             |
| DNA                          | E. coli RNA     | 53                            |
| DNA                          |                 | 31                            |
| E. coli RNA                  |                 | 0                             |

Uracil Inhibition Kinetics—Fig. 9 shows the results of an examination of the kinetics of uracil inhibition of wheat germ glycosylase activity. The pattern obtained is typical of a noncompetitive mode of inhibition by uracil with a family of straight lines converging to a common intercept on the 1/[S] axis. Therefore, uracil may be able to combine with either the free glycosylase or the glycosylase-DNA complex. The apparent K values of the uninhibited reaction with T5 DNA substrate is 8.3 \( \times 10^{-8} \) M and the K is 4.0 \( \times 10^{-1} \) M.

Completeness of Reaction—To determine whether the distance between uracils in the DNA substrate affected the extent of reaction, the double-stranded co-polymer poly(dA-dT) into which uracil had been incorporated to varying degrees (see Experimental Procedures) was exposed to the wheat germ uracil-DNA glycosylase. Fig. 10 shows the ability of the enzyme to remove all available uracil from three poly(dA-dT) substrates in which 5, 50, and 100% of the thymine residues in newly synthesized regions have been replaced by uracil. In all three substrates, a large majority of the available uracil was removed by enzyme action at the reaction's completion. The small differences in extent of reaction between the three substrates are probably due to the presence of small oligonucleotides refractory to uracil-DNA glycosylase attack. When substrates were not purified by gel filtration prior to enzyme assay, substantial differences in apparent extent of reaction were observed, almost certainly due to the presence of such oligonucleotides.

Fig. 8. Inhibition by DNA and RNA. Calf thymus DNA (1 mg/ml) or wheat germ RNA (1.2 mg/ml) was added to the standard assay medium except that the T5 DNA substrate was replaced by labeled calf thymus DNA. □—□, wheat germ RNA; ○—○, calf thymus DNA.

Fig. 9. Double reciprocal plot of uracil inhibition of glycosylase activity. The substrate used was double-stranded T5 uracil-containing DNA.

Fig. 10. Double reciprocal plot of uracil inhibition of glycosylase activity. The substrate used was double-stranded T5 uracil-containing DNA.
A wheat germ uracil-DNA glycosylase has been isolated and purified over 1000-fold. This is the first enzyme of its class to be isolated from a higher plant source. Several facets of its activity have been examined which included assessing the inhibitory effect of a large variety of uracil analogues, comparing the effect of T5-induced E. coli uracil-DNA glycosylase inhibitor on the wheat germ and bacterial glycosyldases, determining a number of physical characteristics of this higher plant enzyme, and examining the effect of uracil distribution along a DNA substrate on the ability of the wheat germ glycosylase to remove that uracil from the substrate.

Of the 14 uracil analogues tested, only 6-aminouracil and 5-azauracil elicited inhibition comparable to uracil itself at 1 mM concentrations. 5-Aminouracil, 6-azauracil, and several other structurally similar pyrimidines with modifications at the 5 or 6 position of the ring had no effect. It is intriguing that a position alteration of the ring nitrogen in 5-azauracil from the 5 to 6 positions totally abolishes the inhibitory effect of this analogue, whereas if the amino group at the 6 position of 6-aminouracil is shifted to the 5 position, inhibition is lost. Clearly, whatever charge or steric constraints govern recognition of the analogues at the site of inhibition are exacting.

This agrees with recent work describing a uracil-DNA glycosylase purified from HeLa cells (11) which was also significantly inhibited only by uracil and these two uracil analogues and very slightly by 5-aminouracil. Comparable, although less extensive, studies have shown that E. coli glycosylase is uninhibited by 5-aminouracil (6) and a human blast cell glycosylase is unaffected by 5-fluourouracil or 6-azauracil (8). This very high specificity is expected as it prevents removal of thymine and cytosine from DNA and uracil from RNA.

Although both wheat germ and E. coli uracil-DNA glycosylase are inhibited by uracil, the response of these enzymes to T5-induced uracil-DNA glycosylase inhibitor is strikingly different, showing that there are at least some structural differences between these two examples of eukaryotic and prokaryotic repair activities preventing effective interaction of the inhibitor with one, but allowing it with the other.

The size of the wheat germ uracil-DNA glycosylase appears similar to that of all bacterial and human uracil-DNA glycosylases examined so far. The molecular weight, determined both by gel filtration and glycerol gradient centrifugation, is approximately 27,000, slightly larger than prokaryotic enzymes (6, 7), very similar to the Seccharomyces cerevisiae glycosylase (20), and smaller than the human blast cell enzyme (8).

The wheat germ glycosylase displays a broad pH optimum and is not overly sensitive to either ionic or nonionic metal ion chelators such as EDTA or 8-hydroxyquinoline. The inhibition caused by high concentrations of EDTA is probably due to the ionic strength of this chelator rather than its metal ion-binding properties because addition of MgCl₂ only further decreases enzymatic activity. The E. coli and HeLa enzymes also are notably affected by low levels of EDTA as shown by Lindahl et al. (6) and Krokan and Wittwer (11), respectively. Therefore, uracil-DNA glycosylases are probably not metalloenzymes.

The inhibitory effects of mono and divalent cations at relatively high concentration on wheat germ glycosylase activity are also accounted for by increased ionic strength of the reaction medium. It is known that as salt concentrations increase, the stability of the DNA duplex increases with a concomitant decrease in nucleic acid-protein complex stability for single-stranded DNA-binding proteins (21, 22), perhaps explaining the uniform falloff in uracil-DNA glycosylase activity observed with increased monovalent, divalent, and ionic chelator concentration.

Some clues about the mode of substrate recognition of the wheat germ glycosylase are given by its inability to release uracil from the mononucleotide dUMP or dUTP as well as the generally similar interaction of RNA and DNA with the enzyme implied by the parallel inhibition patterns for the two polynucleotides. This could indicate that the substrate recognition of the glycosylase and binding site has a bipartite structure such that it is relatively nonspecific for polynucleic acids, being capable of binding single-stranded DNA and double-stranded DNA or RNA, but has a subsite which is extremely specific for uracil bases attached to 2′-deoxyribose residues on that polynucleotide as evidenced by the analogue studies. The noncompetitive inhibition pattern seen on T5 DNA is consistent with the enzyme having two sites important for enzymatic activity.

The Kᵢ, calculated for the wheat germ uracil-DNA glycosylase (8.3 × 10⁻⁴ M) is similar to the values obtained for E. coli, B. subtilis, and human lymphoblast enzymes, which vary from 3.9 × 10⁻⁴ to 1.0 × 10⁻³ M (4). The apparent Kᵢ of the wheat germ glycosylase (4.0 × 10⁻⁴ M) derived from a double reciprocal noncompetitive uracil inhibition pattern is also within the range of values found for other enzymes of this class. Thus, the kinetic characteristics of the wheat germ enzyme are like other uracil-DNA glycosylases for which these characteristics have been examined.

By examining the effect of amount of uracil in the DNA substrate on the ability of the wheat germ glycosylase to excise the base, it was demonstrated that the extent of uracil removal is unaffected by uracil proximity in DNA substrates containing uracil residues as close as every other base or as far apart as one base in every 40. In both cases, the substrate underwent near total removal of available uracil, indicating that the enzyme requires at most one base between uracils excised on a poly(dA-dT) substrate.

Further characterization of the wheat germ enzyme’s active site and mechanism of action will require more direct classical methods of enzyme study and perhaps sequencing of a glycosylase gene allowing the primary structure of the protein to be determined.

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Wheat Germ Uracil Glycosylase

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