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Specificity of the Bacteriophage PBS2 Induced Inhibitor of Uracil-DNA Glycosylase†

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ABSTRACT: The purified PBS2 phage-coded inhibitor of uracil-DNA glycosylase (Ura-DNA glycosylase) from *Bacillus subtilis* has been tested for its ability to inhibit this enzyme isolated from other prokaryotic and from eukaryotic sources. In addition, the inhibitor has been assayed for its effect on DNA glycosylases specific for other base residues in DNA. The data indicate that Ura-DNA glycosylases from a variety of sources are equally sensitive to inhibition by the inhibitor. DNA glycosylases specific for base residues in DNA other than uracil are not inhibited by the PBS2-coded inhibitor.

The DNA glycosylases comprise a group of enzymes that initiate repair of DNA by cleavage of the glycosyl bond of damaged or inappropriate bases in DNA. To date, several distinct DNA glycosylases, each with an apparently stringent substrate specificity, have been isolated from both prokaryotic and eukaryotic organisms [for recent reviews, see Lindahl (1979) and Duncan (1981)]. These include separate glycosylases acting on DNA containing uracil (Lindahl et al., 1977; Cone et al., 1977; Talpaert-Borle et al., 1979; Caradonna & Cheng, 1980; Cone & Friedberg, 1981), hypoxanthine (Karran & Lindahl, 1978; Karran, 1981), 3-methyladenine (Laval, 1977; Riazuddin & Lindahl, 1978), 7-methylguanine residues containing a cleaved imidazole ring [2,6-diamino-4-hydroxy-5-(*N*-methylformamido)pyrimidine (FaPy)[1] (Chetsanga & Lindahl, 1979), urea (Breimer & Lindahl, 1981), ring-saturated thymine monoadducts (Demple & Linn, 1980), pyrimidine dimers (Haseltine et al., 1980; Radany & Friedberg, 1980; Seawell et al., 1980), and intact 7-methylguanine (Cathcart & Goldthwait, 1981; Laval et al., 1981; Margison

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[1] Abbreviations used: Ura-DNA glycosylase, uracil-DNA glycosylase; FaPy, 2,6-diamino-4-hydroxy-5-(*N*-methylformamido)pyrimidine; Hyp-DNA glycosylase, hypoxanthine-DNA glycosylase (Hx in figures); AP, apurinic/apyrimidinic; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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& Pegg, 1981; Singer & Brent, 1981).

As far as is known, all of these enzymes act by cleaving the glycosyl bond linking the affected base to the deoxyribose phosphate residue in a polynucleotide chain, thereby facilitating the release of the damaged or inappropriate base in free form. The resultant apurinic or apyrimidinic (AP) site in DNA can be subsequently repaired by excision of the deoxyribose phosphate residue following incision of the DNA by apurinic/apyrimidinic (AP) endonucleases that are specific for sites of base loss in DNA (Friedberg et al., 1978; 1981; Lindahl, 1979). There is also evidence that apurinic (but not apyrimidinic) sites in DNA may be repaired by direct insertion of the appropriate purine by the catalytic action of DNA purine insertase (Deutsch & Linn, 1979a,b; Livneh et al., 1979).

One of the more extensively characterized DNA glycosylases is the Ura-DNA glycosylase from *Bacillus subtilis* (Cone et al., 1977; Cone & Friedberg, 1981). This organism is the natural host for bacteriophage PBS2, in which uracil normally replaces thymine in DNA (Takahashi & Marmur, 1963). Very early after infection of *B. subtilis*, phage PBS2 expresses several enzyme activities and other proteins which collectively facilitate the incorporation of dUMP rather than TMP into newly synthesized phage DNA (Kahan, 1963; Tomita & Takahashi, 1969; Price & Cook, 1972; Price & Fogt, 1973; Price, 1976). The retention of uracil in PBS2 DNA is achieved by the expression of a phage-coded inhibitor of the host Ura-DNA glycosylase (Friedberg et al., 1975; Katz et al., 1976; Duncan & Warner, 1977). This inhibitor has been extensively purified from PBS2-infected *B. subtilis* (Cone et al., 1980) and shown to be a protein of $M_r \sim 20000$. The exact mechanism of action of the PBS2-induced inhibitor is not yet known, but preliminary data (R. Cone and E. C. Friedberg, unpublished experiments) indicate that it binds to the *B. subtilis* enzyme, thereby inactivating it.

In view of the fundamental similarity of the reaction mechanism of all known DNA glycosylases, it was of interest to use these enzymes to examine the specificity of the PBS2-induced inhibitor of Ura-DNA glycosylase of *B. subtilis*. In this study, we have investigated the ability of this PBS2-induced protein to inhibit in vitro Ura-DNA glycosylases from a variety of biological sources. In addition, we have examined the effect of the purified inhibitor on the activity of three other DNA glycosylases extracted from *Escherichia coli* or calf thymus.

Materials and Methods

Source of Enzymes and Inhibitor. Ura-DNA glycosylase from *B. subtilis* was purified as described by Cone & Friedberg (1981). The preparation used had a specific activity of 1.5×10^8 units/mg of protein. One unit of activity is defined as the amount of enzyme required to liberate 1.0 pmol of uracil in 15 min at 37 °C. Ura-DNA glycosylase from *E. coli* was a gift from Dr. T. Lindahl, University of Goteborg, Sweden. The preparation used was the DNA–agarose fraction (fraction V) (Lindahl et al., 1977). This preparation contained 7.6×10^8 units/mg of protein. Yeast Ura-DNA glycosylase was a gift from Dr. L. Prakash, University of Rochester, Rochester, NY. The fraction used was purified 10800-fold and had a specific activity of 17300 units/mg of protein (B. Crosby and L. Prakash, unpublished experiments). Ura-DNA glycosylase from cultured human KB cells was also examined. The nuclear and mitochondrial activities were present in crude extracts prepared from purified nuclear and mitochondrial fractions as described by Anderson & Friedberg (1980). The nuclear and mitochondrial extracts contained 10.5×10^3 and 46.4×10^3 units of Ura-DNA glycosylase activity per mg of protein, respectively. Hypoxanthine-DNA glycosylase (Hyp-DNA glycosylase) was purified from calf thymus. The preparation used was the DNA–cellulose fraction (fraction V) (Karran, 1981). This preparation contained 243 microunits of Hyp-DNA glycosylase per mg of protein. One unit of activity releases 1 μmol of hypoxanthine per min at 37 °C under standard assay conditions. 3-Methyladenine-DNA glycosylase was prepared from *E. coli* by a slight modification of the procedure described by Riazuddin & Lindahl (1978). The preparation used was approximately 1000-fold purified and had a specific activity of 5000 units/mg of protein. One unit of 3-methyladenine-DNA glycosylase releases 1 μmol of 3-methyladenine per min at 37 °C under standard assay conditions. FaPy-DNA glycosylase was prepared from *E. coli* as described by Chetsanga & Lindahl (1979). The preparation used was the Sephadex G-75 fraction and represented a purification of approximately 20-fold. One unit of FaPy-DNA glycosylase releases 1 μmol of FaPy per min at 37 °C under standard assay conditions.

The PBS2 phage-coded Ura-DNA glycosylase inhibitor was purified from phage-infected *B. subtilis* by the procedure of Cone et al., (1980). The preparation used (fraction V) had a specific activity of 3×10^7 units/mg of protein. One unit of inhibitor inhibits 1 unit of *B. subtilis* Ura-DNA glycosylase assayed under standard conditions.

All enzymes and inhibitors were stored frozen at −196 °C in small aliquots. Enzyme preparations were thawed no more than twice. Inhibitor aliquots were used once and were not refrozen.

Polynucleotide and DNA Substrates. Poly([^{3}H]dU) was prepared by using terminal deoxynucleotidyl transferase as described by Cone et al. (1977). The preparation used had a specific activity of 125 cpm/pmol of uracil. [^{3}H]dITP was made by deaminating [^{3}H]dATP (29 Ci/mol; New England Nuclear Corp.) with fresh nitrous acid as described by Karran (1981). Poly([^{3}H]dI) was synthesized with terminal deoxynucleotidyl transferase as described by Karran & Lindahl (1978). Poly([^{3}H]dI)·poly(dC) was prepared by mixing equimolar amounts of the respective single-stranded homopolymers. The specific activity of the preparation used was 1000 cpm/pmol of dIMP residues. [^{3}H]Dimethyl sulfate (New England Nuclear Corp.; 4.3 Ci/mmol) was used to treat calf thymus DNA. This alkylated DNA had a specific activity of 2100 cpm/pmol of alkylated base. It was used directly to assay 3-methyladenine-DNA glycosylase activity as described by Riazuddin & Lindahl (1978), or after alkali treatment to promote ring opening of 7-methylguanine residues, to assay FaPy-DNA glycosylase as described by Chetsanga & Lindahl (1979).

Enzyme Assays. (*A*) *Ura-DNA Glycosylase.* The standard assay (50 μL) contained 70 mM Hepes–KOH, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, 0.1% bovine serum albumin, and a limiting amount of enzyme, diluted as appropriate just before use in the assay mixture *minus* poly([^{3}H]dU). Reactions were initiated by the addition of poly([^{3}H]dU) (160 pmol; 125 cpm/pmol). After 30 min at 37 °C, reaction tubes were chilled and supplemented with 10 μL of bovine serum albumin (5 mg/mL) and 60 μL of 0.8 M perchloric acid. After 5 min at 0 °C, samples were centrifuged (15000*g* for 5 min), and the radioactivity in 90-μL supernatants was determined in a Triton X containing scintillation cocktail. This assay procedure was used for all ura-DNA glycosylase preparations tested.

(*B*) *Other DNA Glycosylases.* 3-Methyladenine-DNA glycosylase activity was assayed as described by Riazuddin

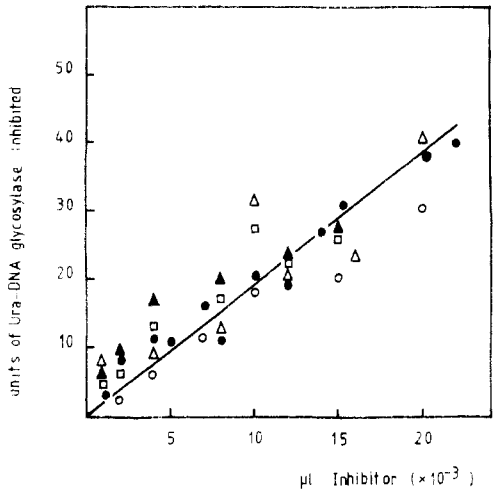

FIGURE 1: Inhibition of Ura-DNA glycosylases from different sources by the PBS2-coded inhibitor. Approximately 50 units of each enzyme was incubated with increasing amounts of inhibitor as shown. Subsequently, the release of acid-soluble material from poly([^{3}H]dU) was measured. A detailed description of the inhibitor assay appears in the text. (●) *B. subtilis*; (○) *E. coli*; (▲) cultured human KB cells, mitochondrial enzyme; (△) cultured human KB cells, nuclear enzyme; (□) yeast.

& Lindahl (1978) and FaPy-DNA glycosylase activity as described by Chetsanga & Lindahl (1979). Hyp-DNA glycosylase was assayed as described by Karran (1981) with the modification that NaCl was included in the standard assay to a final concentration of 0.1 M. Under the assay conditions employed, all DNA glycosylase preparations used were previously shown to release exclusively free base from their respective substrates. Consequently, no specific characterization of the reaction products was carried out in this study.

Assay of Inhibitor Activity. In the standard inhibitor assay, 0–50 units of Ura-DNA glycosylase and varying amounts of inhibitor were combined in a final volume of 25 μL containing 70 mM Hepes–KOH, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% bovine serum albumin. Ura-DNA glycosylase and inhibitor were diluted and mixed immediately before use. After incubation for 5 min at 37 °C, the reaction tubes were returned to ice and supplemented with 25 μL of poly([^{3}H]dU) (160 pmol, 125 cpm/pmol) in 70 mM Hepes–KOH, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% bovine serum albumin. Tubes were capped and returned to 37 °C for a further 30-min incubation. Acid-soluble radioactivity was measured as described above.

Inhibitor assays with other DNA glycosylases were carried out in an identical fashion, using, in general, fixed amounts of the DNA glycosylase under study and variable amounts of inhibitor. For assay of FaPy- and Hyp-DNA glycosylases, the mixtures contained 100 mM NaCl throughout. This ionic strength is optimal for the enzyme activities and has no effect on the inhibitor. 3-Methyladenine-DNA glycosylase was assayed for 20 min following the initial 5-min preincubation with inhibitor. In all these cases, final precipitation was effected with ethanol instead of perchloric acid, as 3-methyladenine, FaPy, and hypoxanthine residues in DNA are somewhat labile under acid conditions.

Results

Inhibition of Ura-DNA Glycosylases. Ura-DNA glycosylases were purified to different extents from a variety of sources. We tested the ability of purified PBS2-coded Ura-DNA glycosylase inhibitor to inhibit these enzymes in vitro. Figure 1 shows that when assayed with purified *B. subtilis*

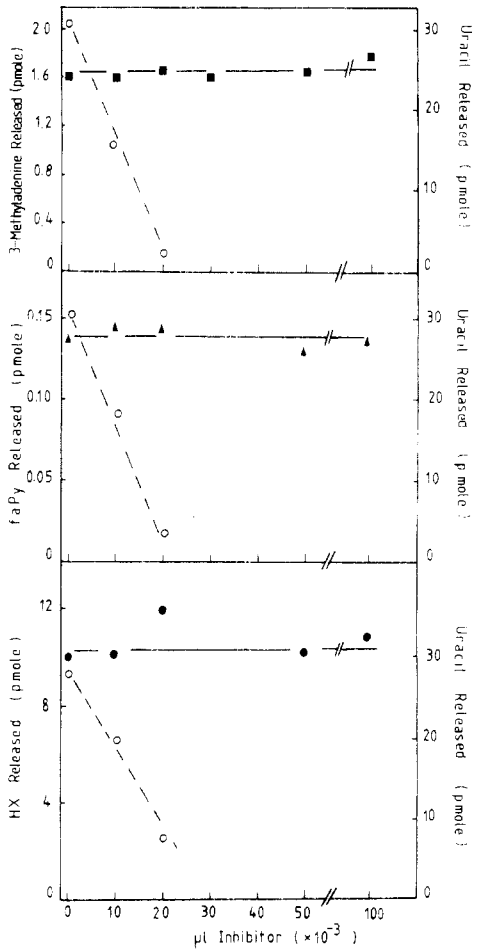

FIGURE 2: Assay of PBS2-coded inhibitor with DNA glycosylases of different specificities. (Lower panel) Highly purified (1000-fold) calf thymus Hyp-DNA glycosylase (0.07 microunit) was incubated with PBS2 inhibitor at the concentrations shown. Release of ethanol-soluble hypoxanthine (●). (Middle panel) Partially purified (10–20-fold) *E. coli* FaPy-DNA glycosylase (8 × 10^{-3} microunit) assayed after incubation with inhibitor as described in the text. Release of ethanol-solubule FaPy (▲). (Upper panel) Highly purified (1000-fold) *E. coli* 3-methyladenine-DNA glycosylase (0.1 microunit) was assayed after incubation with inhibitor as described in the text. Release of ethanol-soluble 3-methyladenine (■). With each set of determinations of inhibitor activity, a parallel standard assay of activity on *B. subtilis* Ura-DNA glycosylase was carried out. These are included in each panel. Release of acid-soluble uracil (○).

Ura-DNA glycosylase, the inhibitor preparation contained 1.9 × 10^3 units of activity/μL. This value is identical with that obtained when assayed with equivalent amounts of Ura-DNA glycosylase from four other sources.

Inhibition of Other DNA Glycosylases. Since the activity of PBS2-induced inhibitor is clearly not confined to Ura-DNA glycosylase from *B. subtilis*, we tested the ability of the inhibitor to interact with DNA glycosylases of different specificities. Figure 2 shows that under conditions which produce pronounced inhibition of Ura-DNA glycosylase, no inhibition of *E. coli* 3-methyladenine-DNA glycosylase, *E. coli* FaPy-DNA glycosylase, or calf thymus Hyp-DNA glycosylase activities were observed.

A possible complication in the interpretation of these experiments is the presence of significant amounts of contaminating Ura-DNA glycosylase in all three DNA glycosylase preparations mentioned above. Two of the preparations, the *E. coli* 3-methyladenine- and the calf thymus Hyp-DNA glycosylases, although highly enriched for the glycosylase under investigation, are not homogeneous and contain readily detectable amounts of Ura-DNA glycosylase. The third prep-

Table I: Effect of Excess PBS2 Inhibitor on Hypoxanthine- and 3-Methyladenine-DNA Glycosylases[a]

enzyme preparation	inhibitor (units)	specific product (pmol)	uracil (pmol)
Hyp-DNA glycosylase (0.06 microunit)	0	9.2	2000
	1.8×10^4	8.5	≤0.2
	7.6×10^4	9.0	≤0.2
3-methyladenine-DNA glycosylase (0.05 microunit)	0	0.9	2500
	1.8×10^4	0.9	≤0.2
	7.6×10^4	0.8	≤0.2

[a] Initial measurements were made of contaminating Ura-DNA glycosylase activity present in the two preparations by using the standard assay described in the text (right-hand column of table). Assays of either Hyp- or 3-methyladenine-DNA glycosylase were then carried out in the presence of concentrations of inhibitor calculated to be in excess of the Ura-DNA glycosylase present by 5–20-fold. The presence of inhibitor in excess was demonstrated directly in a parallel assay in which 40 units of *B. subtilis* Ura-DNA glycosylase was added to the combination shown without detectable release of free uracil. Thus, in the experiment shown, the inhibitor concentration was in excess by ≥40 units.

Table II: Effect of PBS2 Inhibitor Combined with Poly(dU) on Several DNA Glycosylases[a]

enzymes	PBS2 inhibitor (units)	poly(dU) (μM)	product formed (pmol)
3-methyladenine-DNA glycosylase (0.05 microunit)	0	0	1.0
	8	0	0.9
	8	200	1.2
	16	0	1.1
	16	200	1.1
FaPy-DNA glycosylase (0.02 microunit)	0	0	0.59
	8	0	0.66
	8	200	0.59
	16	0	0.71
	16	200	0.61
Hyp-DNA glycosylase (0.06 microunit)	0	0	9.5
	8	0	9.6
	8	200	6.1
	16	0	9.1
	16	200	6.1
	0	200	6.0

[a] Calf thymus Hyp-DNA glycosylase, *E. coli* FaPy-DNA glycosylase, and *E. coli* 3-methyladenine-DNA glycosylase were assayed in the presence or absence of PBS2 inhibitor and nonradioactive poly(dU). Assay conditions are as described in the text.

aration is a relatively crude fraction; it is about 20-fold purified and is not significantly enriched for FaPy-DNA glycosylase with respect to Ura-DNA glycosylase. We considered the possibility that the affinity of each contaminating Ura-DNA glycosylase for the inhibitor may be greater than that of the DNA glycosylase being assayed. Thus, a "preferred complex" might be formed between inhibitor and Ura-DNA glycosylase, leaving insufficient free inhibitor to interact with the DNA glycosylase under investigation. This possibility was excluded in two ways. First, the assays were performed in the presence of levels of inhibitor sufficient to inhibit totally all contaminating Ura-DNA glycosylase and still be in excess. The inhibitor was shown to be in excess by its ability to inhibit exogenously added *B. subtilis* Ura-DNA glycosylase (Table I). This table shows that for the two DNA glycosylase preparations tested, the presence of excess inhibitor did not alter the activity of either Hyp- or 3-methyladenine-DNA glycosylase. In a second series of experiments, assays of either the Hyp-, the 3-methyladenine-, or the FaPy-DNA glycosylases were carried out in the presence of a large excess of nonradioactive poly(dU). This should decrease the amount of any "preferred complex" formed, as inhibitor and poly(dU) would compete for available Ura-DNA glycosylase. Inclusion of poly(dU) up to 200 μM nucleotide residues in the assay mixture in the presence of inhibitor did not result in a significantly increased inhibition of Hyp-, 3-methyladenine-, or FaPy-DNA glycosylases. The slight inhibition of Hyp-DNA glycosylase shown in Table II is due to a nonspecific effect of poly(dU) on this enzyme, as Hyp-DNA glycosylase is known to be inhibited by single-stranded DNA (Karran & Lindahl, 1978).

Discussion

Ura-DNA glycosylases have been isolated from a variety of sources and are apparently widely distributed in nature. The identical level of inhibition observed with Ura-DNA glycosylases from *B. subtilis*, *E. coli*, yeast, and two different intracellular locations in cultured human KB cells strongly suggests that the mode of substrate recognition and the mechanism of action of all Ura-DNA glycosylases are similar, if not identical, and indicates a significant evolutionary conservation of this enzyme. Furthermore, the data demonstrating the absence of a detectable influence of the inhibitor on a number of other DNA glycosylases provide direct evidence that the inhibition of Ura-DNA glycosylases by the PBS2-induced protein is a highly specific reaction. The details of this reaction are currently under investigation using highly purified PBS2-induced inhibitor and homogeneous Ura-DNA glycosylase from *B. subtilis* (E. Yang, J. D. Love, and E. C. Friedberg, unpublished experiments).

The specificity of the PBS2-induced inhibitor for Ura-DNA glycosylase is totally consistent with its postulated biological function, i.e., the preservation of uracil incorporated into DNA during semiconservative PBS2 DNA synthesis. DNA glycosylases have been implicated in the repair of a variety of forms of DNA damage, including alkylation, spontaneous base damage, and possibly ionizing radiation induced base damage (Lindahl, 1979). In some systems, DNA glycosylases also participate in the excision of UV-induced pyrimidine dimers from DNA (Haseltine et al., 1980; Radany & Friedberg, 1980; Seawell et al., 1980). While the existence of a phage-coded inhibitor for the Ura-DNA glycosylase of the host is clearly essential for the maintenance of the viability of phage PBS2, it is equally clear that any cross-inhibition of other DNA glycosylases would place the phage at risk to the lethal and mutagenic action of other DNA damaging agents.

An inhibitor of Ura-DNA glycosylase has also been reported in extracts of bacteriophage T5 infected *E. coli* (Warner et al., 1980) even though this phage contains thymine in its DNA as a principal pyrimidine. It is possible that very small amounts of uracil are normally present in this phage genome and are required for specific functions. Alternatively, the T5-induced inhibitor may have other functions. For example, it has been shown that following infection of *E. coli* by phage T5, inhibition of the *E. coli* ATP-dependent UV DNA incising activity results (Strike, 1978). This result is not observed following infection with phages T3 or T7 (Strike, 1978). Thus, it is possible that the phage T5 induced protein is also an inhibitor of one or more of the *E. coli uvr*A, *uvr*B, or *uvr*C gene products, or of the complex formed by association of these products. Studies on the T5 inhibitor of Ura-DNA glycosylase are currently in progress to determine its substrate specificity and to compare its properties with those of the PBS2-coded inhibitor.

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