Excision of uracil residues in DNA: mechanism of action of *Escherichia coli* and *Micrococcus luteus* uracil-DNA glycosylases

Anne-Marie Delort*, Anne-Marie Duplaa, Didier Molko and Robert Teoule
Laboratoires de Chimie, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires de
Grenoble, 85X, F.38041 Grenoble Cedex, France, and

Jean-Pierre Leblanc and Jacques Laval
Unité de Biochimie et Enzymologie, Institut Gustave Roussy, F.92805 Villejuif Cedex, France

Received 6 December 1984; Accepted 3 January 1985

ABSTRACT

Various octadeoxynucleotides containing uracil at different positions were synthesized and submitted to the action of *Escherichia coli* and *Micrococcus luteus* uracil-DNA glycosylases. A uracil residue situated at the 5'-end was excised by the *M.luteus* enzyme but not by the *E.coli* one. Uracil residues located at the ultimate and penultimate positions at the 3'-end were not cleaved by either enzymes. At the other central positions, uracil was eliminated with different initial velocities. Single stranded ϕ X 174 DNA fragments were used to study the influence of the sequence. Cytosine bases were deaminated to give uracil by bisulfite treatment. It was shown that the initial excision velocity of two vicinal uracil residues was decreased. The same observation was made for two uracils separated by one base. A hypothetical scheme is suggested to explain the mechanism of action of uracil-DNA glycosylases.

INTRODUCTION

Uracil residues can appear in DNA either during replication by incorporation of dUTP instead of dTTP (1-2) or by deamination of cytosine residues. In DNA, uracil is removed by a specific enzyme, the uracil-DNA glycosylase which releases a free uracil and yields an apyrimidic site (AP-site). This enzymatic activity has been found in both prokaryotes or eukaryotes. Although most of their properties appear to be similar [1,2], a detailed comparison of the properties of the enzymes from *Escherichia coli*, *Micrococcus luteus* and H_4 cells (nuclear and cytoplasmic enzymes) shows that they behave differently [3]. A complete removal of uracil, resulting from cytosine deamination, is of utmost importance for the cells. If this lesion is not removed, a transition mutation (G-C $\rightarrow$ A-T) appears after the next round of replication [4,5]. Data concerning the overall action of uracil-DNA glycosylases are available [1,2]. They are small proteins, which act two fold faster on dUMP residues in single stranded DNA than those of double stranded DNA (1-3). The *E.coli* enzyme does not act by phosphorolysis [6] and the *M.luteus* enzyme is not a

processive enzyme [7]. However, very little attention has been given to the excision of uracil residues, depending on their position in the DNA chains. In order to study the enzyme requirements at the molecular level, we have compared the initial rate of excision of uracil located at different positions of synthetic octanucleotides. Using long single-stranded DNA fragments, we have also investigated the influence of the neighbouring bases on uracil excision.

MATERIAL AND METHODS

Materials

ØX174 RF DNA Hae III digest was purchased from New England Biolabs, Intestine alkaline phosphatase, venom phosphodiesterase from Boehringer-Mannheim, T_4 polynucleotide kinase and gamma ^{32}P -ATP (3000 Ci/mmmole) from Amersham.

Preparation of 5'-end labeled single stranded DNA fragments containing dUMP residues

Strands separation of ^{32}P -5'-end labeled DNA : Three μg of ØX174 RF DNA Hae III digest were labeled according to Chaconas and Van de Sande [8]. The DNA fragments concerned are base numbered according to Sanger et al [9] beginning at the cleavage site Z of Hae III restriction fragments and extending in the 5' to 3' direction of the viral(+) or complementary(-) strands. After electrophoresis on a 6% polyacrylamide gel, the fragments were excised and the DNA was extracted by either electro-elution or elution diffusion, followed by DE-52 cellulose (Whatman) column chromatography and ethanol precipitation [10]. The salt-free 5'-end labeled DNA fragment were dissolved in 50 μl of 30 % (v/v) DMSO (dimethylsulfoxide), containing 0.5 mM EDTA and heat-denaturated for 3 minutes at 90°C. The purification of single stranded fragments was performed by electrophoresis on a 6 % polyacrylamide gel in 50 mM tris-borate (pH 8.3), 1 mM EDTA. The migration was allowed to occur for 16 to 18 hrs at 150 V, then the ^{32}P -single stranded DNA fragments were recovered and purified as above.

Deamination of cytosine in DNA fragments : The specific deamination of deoxycytidine to deoxyuridine of ØX 174 single-stranded DNA fragments was achieved by sodium bisulfite treatment according to Kozak [11]. The resulting mixture was dialysed overnight against distilled water and lyophilized.

Analysis of the modified DNA fragments : Mutated fragments were digested by a mixture of venom phosphodiesterase (4 units) in 10 μl of 2.0 M Tris-HCl, 2.5 mM MgCl_2 (pH 9.0) at 37°C for 3 hours. The enzyme was

eliminated by centrifugation (12,000 g for 15 min) The supernatant, layered on PEI cellulose sheet was first migrated in 1M lithium chloride, dried, and then migrated in 0.5 M ammonium formate (pH 4.5) in the second dimension. dGMP, dUMP, dCMP, dTMP, dAMP were visualized by fluorescence under UV light (254 nm).

Preparation of 5'-end 32 P-labeled synthetic oligodeoxyribonucleotides

d(GTACGTAC), d(GTAUGTAC) were prepared in solution by the phosphotriester method [12]. d(GTACUTAC), d(GTACGTUC), d(GTACGUAC) were synthesized on solid support by the phosphotriester method [13] d(UTACGTAC), d(GUACGTAC), d(GTUCGTAC) were prepared on solid support by a mixed phosphotriester-phosphoramidite method [13,14,15].

The oligomers were purified by reverse phase high performance liquid chromatography using Nucleosil C-18 column with an acetonitrile gradient in 0.1 M triethylammonium acetate (pH 7.0). The procedure was repeated twice. The sequences of the synthetic octamers were confirmed by the Maxam and Gilbert method [16] modified by Banaszuk et al [17].

d(GTAUGTAU) was obtained by specific deamination of d(GTACGTAC) using sodium bisulfite [11] The conversion to uracil was checked as described above by TLC on PEI cellulose.

Aliquots of 500 μ moles of oligonucleotides ($\sim 10^{-2}$ A₂₅₄) were 32 P-5'-end labeled [18,19], then the 5'-end labeled octanucleotides were separated from the 5'-hydroxyl terminated-ones by reversed phase high performance liquid chromatography as previously described [19].

Enzymatic reactions

Two uracil-DNA glycosylases of different origins were used : the M.luteus enzyme was the homogeneous fraction isolated by Leblanc et al. [7] and the E.coli enzyme was purified to homogeneity using the same procedure [3].

M.luteus assay conditions [7] : 32 P-5' end labeled DNA fragments were taken up into a buffered solution (7 mM Hepes NaOH, 35.5 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol pH 6.8) with uracil-DNA glycosylase diluted if necessary in the standard mixture where DNA was omitted, but supplemented with bovine serum albumin (200 μ g/ml). After incubation at 37°C for increasing periods of time as stated in the legends of figures, 10 μ l aliquots were withdrawn and added to 10 μ l of a solution containing 10 mM NaCl, 20 μ g of uracil and 10 μ g of calf thymus DNA and kept at 0°C, to stop the reaction.

E.coli assay conditions [6] : 32 P 5'-end labeled DNA fragments were taken

up into the standard reaction mixture containing 70 mM Hepes KOH (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol and uracil-DNA glycosylase, diluted if necessary in 0.3 M NaCl, 50 mM Hepes KOH (pH 7.4) 1 mM EDTA, 1 mM dithiothreitol, 0.01 % bovine serum albumine. After incubation at 37°C for increasing periods of time, the reaction was stopped as described for M.luteus.

Uracil excision from synthetic oligonucleotides and analysis of the reaction by sequencing : Except in the case of d(GTAUGTAU), equimolar amounts of d(GTAUGTAC) and of the octamer to be studied, (i.e. equivalent amounts of radioactivity) were mixed. The ^{32}P -5'-end labeled oligonucleotides mixture ($\sim 1.200 \cdot 10^6$ cpm in 60 μl) was digested by 0.017 units of either M.luteus or E.coli uracil-DNA glycosylase at 37°C under the reaction conditions described above.

After incubation with the enzyme, the reaction was stopped as already described. Then the samples were supplemented with 5 μg of tRNA. After ethanol precipitation and drying, the pellets were dissolved in 100 μl of freshly diluted 1 M piperidine to break phosphodiester bonds at the apyrimidic sites as described in the Maxam and Gilbert sequencing procedure [16]. Proper controls were run at the same time.

All samples were heated at 90°C for 1 min in loading buffer (3 μl of 80 % formamide (v/v), 10 mM NaOH, 1 mM EDTA, 0.1 % xylene cyanol w/v), chilled and quickly loaded on a sequencing gel (0.4 mm thick, 40 cm long, 25 % polyacrylamide, 1/20 cross-linked, 7 M urea). Electrophoresis was carried out at 2500 V for 2 hours in 50 mM tris-borate, 1 mM EDTA (pH 8.3). The oligonucleotides spots located by autoradiography, were excised and counted by Cerenkov effect.

Enzymatic uracil excision from modified DNA ϕX 174 fragments : ^{32}P 5'-end labeled single stranded ϕX 174 DNA restriction fragments, deaminated with sodium bisulfite were dissolved in 9 μl of the adequate buffer and 0.0017 units (1 μl) of uracil-DNA glycosylase (from either E.coli or M.luteus) . The mixtures were incubated at 37°C. After 5 min, 10 μl of stop solution were added and the mixtures were chilled in ice. Then they were treated as described above for synthetic oligonucleotides. Electrophoresis was carried out in 0.4 mm thick, 40 cm long, 8 % polyacrylamide slabs, 1/20 cross linked containing 50 % urea, at 1300 V, for 3 hours in 50 mM Tris borate, 1 mM EDTA (pH 8.3). The autoradiography of the gel with no screen Kodak film for 96 hours, revealed spots corresponding to uracil excision by uracil-DNA glycosylase. The relative intensities of the spots were

measured by optical densitometry at 540 nm on a Transidyne 2955 RFF scanning densitometer. The data were recorded as absorbance in relation to the film base and the peak areas were measured with a Phymetron Numonics 1210 integrator.

RESULTS

In order to study the mechanism of action of uracil DNA glycosylases, synthetic oligonucleotides containing uracil at different positions were prepared by phosphotriester and phosphoramidite methods. The following products d(UTACGTAC), d(GUACGTAC), d(GTUCGTAC), d(GTAUGTAC), d(GTACUTAC), d(GTACGUAC), d(GTACGTUC), d(GTACGTAC) were synthesized chemically and sequenced. d(GTAUGTAU) was obtained by bisulfite deamination of d(GTACGTAC). ØX 174 single stranded DNA fragments treated with sodium bisulfite were analysed to check the conversion of cytosine into uracil. An aliquot of each sample was treated by venom phosphodiesterase and the reaction mixture was chromatographed on PEI cellulose sheets which showed a cytosine deamination upper than 98 %.

Sequencing by the Maxam and Gilbert procedure for cytosine positions gives the same results if AP sites are generated chemically from cytosine residues or enzymatically from uracil residues obtained by deamination

In most cases, the activity of uracil-DNA glycosylase is determined by the release of free radioactive uracil. In this work, we used the method in which the apyrimidic sites, produced by the excision of uracil residues, are measured [16]. Maxam and Gilbert showed that heating a DNA fragment containing a AP site in 1.0 M piperidine at 90°C yields quantitatively a DNA chain rupture. As shown in figure 1, lane 1, the sequence of the ØX 174 fragment at cytosine residues was obtained by the chemical cleavage reaction of Maxam and Gilbert. If the DNA fragment is deaminated and contains uracil at the position of cytosine, the same pattern is observed (Lane 2). The treatment of the ØX 174 fragment containing dUMP residues with uracil-DNA glycosylase, followed by piperidine hydrolysis and separation by electrophoresis on a sequencing gel showed bands at positions corresponding to cytosine but not at other positions (Lane 4). When the DNA was not treated with bisulfite but incubated with uracil-DNA glycosylase, the DNA chain is not cleaved (Lane 3) showing that the enzyme was not contaminated by some non specific nuclease.

Therefore, the depyrimidinisation of cytosine by either hydrazine, as

Figure 1 : Autoradiogram of a ^{32}P sequencing gel of the 5'-end labeled single stranded Hae III restricted ϕX174 DNA 234 bases long, (-)7 at the restriction site Z (9). Lane 1 is the classical chemical sequencing at C positions. In lane 2, the same reaction was carried out on the same fragment after deamination of cytosines to uracils. In lane 3, the natural fragment was submitted to the action of M.luteus uracil-DNA glycosylase and heated in 1 M piperidine. In lane 4, the fragment was C deaminated to uracil, digested by M.luteus DNA glycosylase and broken at AP sites by heating in piperidine.

in the Maxam-Gilbert procedure, or by uracil-DNA glycosylase excision of uracil leads to the same products since both processes yield AP-sites at the same positions in the oligonucleotides. The AP-sites are further cleaved by the same procedure using piperidine treatment. In conclusion,

Figure 2 - A : Autoradiogram of a 25 % sequencing gel, 7M urea of ^{32}P -5' end labeled octadeoxyribonucleotide mixture d(GTAUGTAC) + d(GTACGUAC) digested by uracil DNA glycosylase from *E.coli*. The indications 1, 2, 5, 10, 20, 40 are the incubation times in minutes. T+p was not digested by the enzyme but treated with piperidine, E was digested by the enzyme for 40 min. but not followed by piperidine treatment.

band 1 : mixture of d(GTAUGTAC) and d(GTACGUAC) ; band 2 : d(GTACGp) resulting from breaking at uracil position in d(GTACGUAC) ; band 3 : d(GTAp) resulting from breaking at uracil position in d(GTAUGTAC).

B : The relative amount of oligomer resulting from enzymatic excision at uracil position and piperidine break at the corresponding AP site are plotted versus incubation times : (○—○—○) is the percentage of d(GTACGp) relative to d(GTACGUAC) ; (△—△—△) is the percentage of d(GTAp) relative to d(GTAUGTAC).

we can follow uracil glycosylase activity by this technique at a specific position in the DNA chain.

Excision of uracil residues from short oligonucleotides by *M.luteus* or *E.coli* uracil-DNA glycosylases.

Preliminary experiments had shown that, when the uracil residues was located in the central part of an oligonucleotide such as d(GTAUGTAC), its excision by uracil-DNA glycosylase was as efficient as that of uracil in longer chain. Therefore the oligonucleotide d(GTAUGTAC) was used as internal reference in samples containing oligonucleotides studied. Equimolar mixtures of d(GTAUGTAC) and of the octamers to be studied were treated by either *M.luteus* or *E.coli* enzymes.

Figure 3 : Excision by *E.coli* uracil-DNA glycosylase of uracil located at the 5'-end. Autoradiogram of a 25% sequencing gel, 7 M urea of [32 P]-5'-end -labeled octadeoxyribonucleotides mixture d(GTAUGTAC) + d(UTACGTAC) digested by uracil-DNA glycosylase from *E.coli* (A) as described in experimental procedure. The indications 1', 2', 5', 10', 20', 40' are the incubation times, T was not treated by the enzyme and piperidine, T+p was not digested by the enzyme but treated with piperidine, E was digested by the enzyme but not followed by piperidine treatment. C is base specific chemical cleavage of cytosine and uracil. Pi is the position of inorganic phosphate.

The figure 2 shows the kinetic excision of uracil residues by the *E.coli* enzyme in the antepenultimate 3'OH position d(GTACGUAC) in relation to the reference compound. The upper band of figure 2A corresponds to the equimolar mixture of 32 P-5'-end labeled d(GTAUGTAC) and d(GTACGUAC). Band 2 consists in d[32 P]-GTACGp and band 3 in d[32 P]-GTAp. They result from the scission of the DNA chain at position of AP-sites and, therefore, their respective radioactivities measure the amounts of uracil previously excised by the uracil-DNA glycosylase in the two substrates. The use of an internal standard allows a better precision in the comparison of the various substrates. Figure 2B shows the time course of uracil excision using, as substrate, d(GTACGUAC) and d(GTAUGTAC). It should be noted that in figure 2A the controls (Lane E and T+P) show that the action of the enzyme alone, or of the piperidine alone, does not break the sugar-phosphate skeleton. We can conclude that the enzyme does not contain any contaminating single stranded endonuclease, exonuclease or AP-endonuclease. The enzyme is not contaminated by 5'specific or non specific phosphatase (see below Figure 3). The same purity was observed for the uracil DNA glycosylase from *M.luteus*. On the other hand, piperidine does not cleave oligonucleotides at dUMP residues, but reveals only uracil-DNA glycosylase action.

Table 1 : Relative initial rates of uracil excision by E.coli and M.luteus for different uracil substituted octamers. The reference sample is dGTAUGTAC. (*) precision : ± 0.05

Substrate	E.Coli (*)	M. luteus (*)
d(UTACGTAC)	0.	1.20
d(GUACGTAC)	1.25	0.65
d(GTUCGTAC)	0.40	0.36
d(GTAUGTAC)	1.00	1.00
d(GTACUTAC)	1.25	1.13
d(GTACGUAC)	0.50	0.76
d(GTACGTUC)	0.0	0.0
d(GTAUGTAU)	0.0	0.0

The relative initial velocities of the enzymes acting on the various oligomers studied : d(UTACGTAC), d(GUACGTAC), d(GTUCGTAC), d(GTAUGTAC), d(GTACUTAC), d(GTACGUAC), d(GTACGTUC) and d(GTAUGTAU) are in Table I.

For both enzymes, an uracil adjacent to the 3'OH and/or in the penultimate position is not excised. The lack of excision of uracil residues is not due to T and C adjacent bases, since it is excised from the octamer d(GTUCGTAC). An important difference in the specificity of the two DNA-glycosylases is observed when they act on dUMP residues adjacent to the 5'-phosphate terminal : the M.luteus enzyme remains fully active while the E.coli enzyme is completely inactive. All the uracil residues located in central positions are excised, although with some differences in the initial velocity. These results suggested that the bases adjacent to uracil may have some influence on its excision. This point was further investigated using deaminated ØX 174 DNA fragments.

The lack of activity of the E.coli enzyme dUMP residues located at the ^{32}P -5'-end was further investigated. We ruled out the possibility of a phosphatase removing the 5'-phosphate in d(UTACGTAC) fragment or in the d(□TACGT) fragment (□ is the symbol chosen for AP-site). If such had been the case, a band of inorganic phosphate should have appeared. But it barely did not if at all (Figure 3). Quantification of P_i showed that it accounted for less than 0.1 % of the total recovered radioactivity. Half of the total radioactivity was found at the octamer position and the other half at the position of dGTAp which allowed us to conclude that if the enzyme can excise uracil at the 5'-end position, its rate is less than 0.1 % of that observed for the excision of uracil from the control octamer.

The study of the excision of uracil residues located at the penultimate position on the 3'OH side of the molecule showed that neither the *E.coli* nor the *M.luteus* enzyme can eliminate it. Even when the autoradiographies were purposely overexposed, no band was detectable at the position of d(GTAUGTp) (data not shown).

The synthetic model used to study the excision of uracil located at the 3'-end was d(GTAUGTAU), which was obtained by deamination of d(GTACGTAC). With this procedure it was no need to prepare a special solid phase support starting with uracil on the first protected base, in order to carry out the chemical synthesis of this oligodeoxynucleotide. In this case, the reference uracil was inside the molecule. It implied that at the beginning of the enzymatic reaction, the intensity of the spots observed represented the relative affinity of the enzyme for the two sites in the molecule, yielding d(GTAUGTAp) and d(GTAp). As the time of incubation increased, d(GTAUGTAp) would be further cleaved to give d(GTAp), and at the end of the reaction, only one spot, d(GTAp) should be observed. In fact, in this experiment even when the autoradiography was overexposed, the spot corresponding to d(GTAUGTAp) during the initial period of digestion was not observed. We can conclude that uracil located at the 3'end is not excised, or if it is, its excision velocity is less than 0.1% of that of the reference uracil (data not shown).

In conclusion, uracil residues, located in the penultimate or ultimate 3'OH position are not excised. This is true for both enzymes studied. Enzymatic excision of uracil in natural single stranded fragments containing uracil in place of cytosine

Figure 4 shows autoradiograms of sequencing gel electrophoresis of different fragments ((+)8, (+)9, (-)7) of ϕ X 174 DNA deaminated by sodium bisulfite, excised by uracil-DNA glycosylase and treated by piperidine.

Figure 4 : Autoradiograms of 8 % sequencing gels of ³²P-5'-end labeled single stranded Hae III restricted fragments of ϕ X 174 deaminated by sodium bisulfite, treated with uracil-DNA-glycosylase, followed by heating in piperidine as described in experimental procedures. (+)9, (+)8, (-)7 are 118, 194 and 234 base long fragments, according to the nomenclature given by Sanger et al [9] at the restriction site Z : a) (+)8 fragment from position 1009 to 1173 digested by uracil DNA glycosylase from *M.luteus*. b) (+)8 fragment from position 1045 to 1173 digested by uracil DNA glycosylase from *M.luteus*. c) (+)9 fragment from position 4772 to 4866 digested by uracil DNA glycosylase from *M.luteus*. d) (-)7 fragment from position 630 to 435 : lane 1 : base specific chemical cleavage of uracil, lane 2 : digestion by uracil DNA glycosylase from *M.luteus* ; lane 3 : digestion by uracil DNA glycosylase from *E.coli*.

Figure 5 : Pannel I : digestion by uracil-DNA-glycosylase from *M.luteus* ; Pannel II : specific chemical cleavage at uracil position a) partial autoradiogram of a 8 % sequencing gel, 7M urea, of (-)7 fragment from position 632 to 583, 5'end labeled, b) Densitogram (at 540 nm) of the corresponding autoradiogram presented in a . The values written above each peak give the corresponding areas, c) Peak area values measured from densitograms b were plotted versus the corresponding uracil positions ordered from the 5' to the 3'end.

Inspection of these autoradiograms shows that the intensity of the different bands at the positions corresponding to uracil excision is not homogeneous along the sequence. When we consider isolated bases, we observed that intensities are variable, depending on the sequence (for instance, in lane d, the positions 598 and 606 as compared to positions 624, 627, 630 ...). A more precise evaluation of these intensities is given by the densitogram of the autoradiographies. In figure 5 the fragment (-)7 (from 632 to 583 position) treated with hydrazine according to the procedure of Maxam and Gilbert (see figure 5-II) is compared with the same fragment digested with uracil-DNA glycosylase from *M.luteus* (figure 5-I).

In the case of the specific chemical cleavage, all uracils are attacked at random regardless of the base sequence. The observed intensity of the bands must decrease uniformly as a function of the distance of uracil from the 5'-P-end : $y = a x + b$ where y is the intensity value and x is the order of position of the uracils from the ^{32}P -5'-end. This is due to the fact that a fragment containing more than one uracil may be cut again to a shorter fragment and so on. In figure 5-II-c, the areas measured on the densitogram (5-II-b) which correspond to the intensities of the bands on the autoradiogram (5-II-a), were plotted against the positions of the uracil residues ordered from the 5' to the 3'-end. In this case, a straight line is obtained (the correlation coefficient of the least squares method is 0.93). This shows that there is no influence of the neighbouring sequence in the case of the reaction of hydrazine on dUMP residues.

In contrast, inspection of fig. 5-I shows that the intensities of the bands are not homogeneous. The plotting of peaks areas against uracil positions does not give a straight line (correlation coefficient 0.49). When other DNA fragments were examined (autoradiogram of figure 4), the intensities of the bands against uracil position were again no random. In every case no straight line was obtained. We can conclude that uracil-DNA glycosylase does not react at random and that uracil sites are not equivalent. This lack of homogeneity of digestion is observed with both enzymes, M.luteus (lane d-2, figure 4) and E.coli one (lane d-3, figure 4). A comparison of the base sequences adjacent to dUMP residues did not reveal any correlation with the slower excision of uracils. However, it should be noticed (figure 4) that the intensities of the electrophoretic bands are lower when the uracils are vicinal (lane a : positions 1023-24, 1034-35, 1042-43 ; lane b : 1047-49, 1060-61, 1089-90 ; lane c : 4789-90, 4806-07 ; lane d : 589-90). The same decrease in the velocity of excision is also observed when the base is located on the 5'-side (underlined in the text) and when two uracils are separated by only one base : for example, position 617 in the sequence pAAUTUAA (lane d), position 4784 in the sequence pGGAUAUTT (lane c), position 1021 in the sequence pGUGUU (lane a).

DISCUSSION

The present study using model DNA fragments containing uracil gives some information about the mechanism of action of uracil-DNA glycosylase.

By a direct determination, we have shown that uracil DNA glycosylases from both E.coli and M.luteus do not recognize the uracil residues, either in the ultimate or in the penultimate positions at the 3'-end. These findings determined the minimal distance from the 3'-end required for enzyme activity. They suggest that the binding of uracil-DNA glycosylases from E.coli and M.luteus which are small globular proteins requires at least two bases on the 3'-side of the excisable uracil. It was suggested earlier that the uracil-DNA glycosylase from E.coli [2], B.subtilis [21] and Hela cells [22] do not excise uracil when this base is located at the 3'-end.

In the case of M.luteus enzyme, the uracil located at the 5' end is normally excised.

In the case of the E.Coli enzyme, uracil located at the 5'-end is not excised. Together with the result obtained at the 3'-end, this suggests that the enzyme needs two bases at the 3'-side of the excisable uracil, and one base at the 5' side of the uracil in order to achieve excision. We can conclude that four nucleotides including uracil are required. The latter model explains why the smallest single stranded oligomer digested by this enzyme is $d(pU)_4$, as previously reported for E.coli [20] or B-subtilis [21]. To be active, the enzyme needs three or four nucleotides, that is about 21 Å - 28 Å if we consider that the length of the bonds phosphate-sugar-phosphate is about 7 Å (in the S conformation of the sugar) [23]. This result is in a good agreement with the space hindrance of the proteins whose Stokes radii are 23 Å (E.coli) and 20 Å (M.luteus).

The different behavior of the two enzymes with respect to the excision of uracil residues located at the 5'-end may be due to a different mechanism of action. The uracil-DNA glycosylase from E.coli is supposed to be a processive enzyme [20] while the M.luteus enzyme is not [7]. Their mode of binding or the place of their catalytic site may, therefore, be different.

If we consider now the long natural fragments studied, it is clear that consecutive uracils or uracils separated in the 5' direction by only one base, are excised at a lower rate than isolated uracils. This can be explained by the model developed earlier. When one molecule of protein binds to a site, another one cannot bind to a vicinal site if the two sites are not separated by at least two bases. In the case of vicinal uracils, proteins have to bind in turn, so that the overall velocity of

excision is decreased. The decrease may also be due to the presence of an apurinic (AP) site next to an uracil, located in the binding site of the enzyme. Talpaert-Borle *et al* [24] have shown that uracil-DNA glycosylase from calf thymus is inhibited by AP-sites.

It is worth noting that the differences in excision rates reported herein represent initial velocities since they were observed during the initial phase of digestion. At the end of the reaction, all uracils will be excised (except at the end of the DNA fragment). There is no inhibition between the different uracil sites, no matter what the distance between these uracils may be. Therefore, our model of reaction give a possible explanation of the results reported by Blaisdell and Warner (25) who showed the ability of the wheat germ uracil-DNA glycosylase to totally remove uracil from synthetic poly(dA-dT) in which thymine had been replaced by uracil in varying percentages (100%, 50%, 5%).

The experiments reported herein allowed us to observe the early phase of the digestion which reveals in fact the mechanism of action of uracil-DNA glycosylase. The study of short oligonucleotides has shown that internal uracils are normally excised, but that initial velocities of excision are slightly different. This could be due to the influence of sequences surrounding uracil but also to the distance from the end of the oligonucleotide. This influence can also be noticed in autoradiograms and densitograms of ϕ X 174 DNA fragments. We have shown that the intensities of the bands are not linearly correlated with uracil position in the fragment. Consequently, the initial velocities of uracil excision does vary with the sequence base surrounding this base.

For instance isolated uracils on the DNA chain which are not concerned by the problem of the free binding sites or AP sites discussed earlier, have different intensities (see fig. 5-II, fragment (-)7). No simple correlation has been found with the nature of either the two bases surrounding uracil, or with the two bases above uracil. Further experiments would be necessary to conclude clearly.

The *M.luteus* as well as the *E.coli* enzymes recognize better single stranded than double stranded DNA, so lower rate of excision may have been due to reannealed regions. Therefore, we looked for a possible secondary structure of these fragments by computer analysis.

This study of a possible secondary structure is illustrated here by

the (-)7 single strand fragment which sequence is as follows :
3'end ...

```
436
GGAGATAATT UGAGTAAGTU UGAAGAUGGU AAAAUUTAAA TTGGUTTUTA UTAAAGUTAA
                                   a
496
AAGAUTGUTU ATTGTTTUAA AUUTAAUGAT GAUTGGUGAG AGUAUGAGUA GUGAUGUAAU
                                   a'
556
TUUGAAUGUA AATAUUATGU GAUUTGAAAAU ATUUTATGGG AGUGAAAGGA UGAGGAUAAU
                                   b          b'          c
616
TUAAAATAAUG AUGGUAGTAA UGAATAATAU AAAGTAGGGUA GTTGTAAAGTT TGUU
c'                                     669
                                   (-)7 fragment ...5'end
```

The underlined sequences are the possible double-stranded regions if we consider that A=U pairing is equivalent to A=T pairing. However, this structure does not help to explain the decrease in band intensities. For instance, the uracils at position 498 and 606 are in a single strand region and their excision rate is low. Therefore, we can conclude that there really exists an influence of the sequences on the initial uracil excision by uracil DNA-glycosylase. Weiss *et al* [26] reported a uniform decrease in band intensities corresponding to uracil excision by *B.subtilis* enzyme , as a function of fragments lengths. However, they used a different enzyme and their work was not aimed at studying the influence of the sequence on uracil-DNA glycosylase action , but rather at looking for the presence of uracil in human DNA fragments. To test their method, they studied fragments with 10 percent of uracil substitution. In this case, the influence of the steric hindrance could not be detected because the probability of finding two vicinal uracils was too low (1%). Moreover, heating with sodium hydroxide causes the deamination of adenine and creates AP-sites.

In conclusion, the experiments presented here allow us to propose a model for the mechanism of action of uracil-DNA glycosylase. They also suggest that the sequence of the bases surrounding uracil has an influence on the rate of excision. Sequencing methods and synthetic oligodeoxynucleotides are valuable tools to investigate enzymatic actions and to show variations undetectable under other conditions.

*To whom correspondence should be addressed

REFERENCES

1. Laval, J. and Laval, F. (1970), I.A.R.C. Scientific Publications, edit. R. Montesano, H. Bartsch and Z. Tomalis, 27, 53-72.
2. Lindahl, T. (1982) *Ann. Rev. Biochem.*, 51, 61-84.
3. Leblanc, J.P. and Laval, J. (1982) *Biochimie*, 64, 735-738.
4. Arenaz, P. and Sirover, M.A. (1983) *Proc. Natl. Acad. Sci. USA* 80, 5822-5826.
5. Drake, J. and Baltz, R.H. (1976) *Ann. Rev. Biochem.* 45, 11-37.
6. Lindahl, T. Ljungquist, S. Sieberg, W. Nybert, B. and Sperens, B. (1977) *J. Biol. Chem.* 252, 3286-3294.
7. Leblanc, J.P., Martin, B., Cadet, J. and Laval, J. (1982) *J. Biol. Chem.* 257, 3477-3483.
8. Chaconas, G. and Van de Sande, J.H. (1980) *Methods in Enzymology* 65, 76-85.
9. Sanger, F., Air, F.M., Barrell, B.D., Brown, N.J., Coulson, A.R., Fiddes, J.C., Hutchinson III, C.A., Slocombe, P.M. and Smith, M. (1977) *Nature*, 265, 687-695.
10. Smith, H.O. (1980) *Methods in Enzymology* 65, 371-380.
11. Kozak, M. (1980) *J. Mol. Biol.* 144, 291-304.
12. Téoule, R., Derbyshire, R.B., Guy, A., Molko, D. and Roget, A. (1980) *Nucleic Acids Res. Symp. Ser.* 7, 23-37.
13. Efimov, V.A., Reverdatto, S.V. and Chakhmakheva, O.G. (1982) *Nucleic Acids Res.* 10, 6675-6694.
14. McBride, L.J. and Caruthers, M.H. (1983) *Tetrahedron Lett.* 245-248.
15. Molko, D., Delort, A.M. and Téoule, R. (1984) *Nucleic Acids Res. Symp. Ser.* 14, 311-312.
16. Maxam, A.M. and Gilbert, W.A. (1977) *Proc. Natl. Acad. Sci. USA* 74, 560-564.
17. Banaszuk, A.M. Deugan, K.U. Sherwood, J. Michalak, M. and Glick, B.R. (1983) *Anal. Biochem.* 128, 281-286.
18. Richardson, C.C. (1965) *Proc. Natl. Acad. Sci. USA* 54, 158-165.
19. Delort, A.M. Derbyshire, R. Duplaa, A.M. Guy, A. Molko, D. and Téoule, R. (1984) *J. Chromatogr.* 283, 462-467.
20. Lindahl, T. (1974) *Proc. Natl. Acad. Sci. USA*. 71, 3649-3653.
21. Cone, R., Duncan, J., Hamilton, L. and Friedberg, E.L. (1977) *Biochemistry* 16, 3194-3201.
22. Krokan, H. and Wittwer, C.U. (1981) *Nucleic Acids Res.* 9, 2599-2613.
23. Rich, A. Quingley, G.J. and Wang, A.H.J. (1980) in *Nucleic Acids Geometry and Dynamics*, Pergamon Press, 273-288.
24. Talpaert-Borle, M., Campagnari, F. and Creissen, D.M. (1982) *J. Biol. Chem.* 257, 1208-1214.
25. Blaisdell, P. and Warner, H. (1983) *J. Biol. Chem.* 668, 1603-1609.
26. Wiess, R.B. Mineura, K. Henderson, E.E. Ducker, N.J. and de Riel, J.K. (1983) *Biochemistry*, 22, 4501-4507.