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Contrasting effects of single stranded DNA binding protein on the activity of uracil DNA glycosylase from *Escherichia coli* towards different DNA substrates

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ABSTRACT

Excision of uracil from tetraloop hairpins and single stranded ('unstructured') oligodeoxyribonucleotides by *Escherichia coli* uracil DNA glycosylase has been investigated. We show that, compared with a single stranded reference substrate, uracil from the first, second, third and the fourth positions of the loops is excised with highly variable efficiencies of 3.21, 0.37, 5.9 and 66.8%, respectively. More importantly, inclusion of *E.coli* single stranded DNA binding protein (SSB) in the reactions resulted in ~7–140-fold increase in the efficiency of uracil excision from the first, second or the third position in the loop but showed no significant effect on its excision from the fourth position. In contrast, the presence of SSB decreased uracil excision from the single stranded ('unstructured') substrates ~2–3-fold. The kinetic studies show that the increased efficiency of uracil release from the first, second and the third positions of the tetraloops is due to a combination of both the improved substrate binding and a large increase in the catalytic rates. On the other hand, the decreased efficiency of uracil release from the single stranded substrates ('unstructured') is mostly due to the lowering of the catalytic rates. Chemical probing with $KMnO_4$ showed that the presence of SSB resulted in the reduction of cleavage of the nucleotides in the vicinity of dUMP residue in single stranded substrates but their increased susceptibility in the hairpin substrates. We discuss these results to propose that excision of uracil from DNA–SSB complexes by uracil DNA glycosylase involves base flipping. The use of SSB in the various applications of uracil DNA glycosylase is also discussed.

INTRODUCTION

Uracil DNA glycosylase (UDG) initiates uracil excision repair pathway by cleaving the glycosidic bond between uracil and the deoxyribose sugar of DNA. Uracil residues in DNA arise as a result of deamination of cytosine or incorporation of dUMP by DNA polymerase. UDGs characterized so far require no metal ions or other cofactors for their activity (1–4) and can be divided into two groups. A number of diverse proteins such as the cyclin like UDG (5,6), recently discovered dsUDG (7) and other proteins with UDG activity such as glyceraldehyde-3-phosphate dehydrogenase (8) can be classified into one group. The mechanism of uracil excision by this group of proteins is not understood at present. The other group consists of UDGs which show striking similarity in their amino acid sequence from all sources (3). Within this group, *Escherichia coli* UDG (Ung) was the first to be discovered and characterized (9,10), and has been used as a prototype to understand the biochemistry of uracil release (1–4). However, much of the current knowledge on the structural basis of the enzyme action has emerged from the crystal structures of UDGs from HSV-I and human. The crystal structures revealed that the active site grooves of this group of UDGs are also highly conserved and that they bind to the extrahelical or flipped out uracil residues (11–13). Such binding of extrahelical bases was earlier reported for two bacterial DNA methyltransferases and several other DNA repair enzymes (14–16). Recently, the structure of an engineered mutant of human UDG complexed with double stranded DNA showed that the uracil base flipping is achieved by the enzyme mediated 'push' and 'pull' mechanism (13,17). The side chain of the leucine residue from a highly conserved motif HPSPLS (position 272 in human UDG) infiltrates the minor groove and expels ('push') the dUMP residue into the major groove of the double helix. The insertion of the leucine side chain is stabilized by interactions of the phosphate groups with selective amino acids on the surface of UDG. Flipping of the uracil residue from the major groove (as opposed to the base flipping from the minor groove for the recognition by methyltransferases) is then facilitated by the interactions with the side chains of amino acids that form the uracil binding pocket ('pull') (13,17). The glycosidic bond between the uracil and the sugar is cleaved by the attack of a hydroxyl nucleophile on to the deoxyribose C1′ atom. The hydroxyl nucleophile is most likely generated by the activation of a water molecule by the aspartate residue of yet another highly conserved motif GQDPYH (11,13,18,19).

Reconstitution of UDG directed repair pathway in *E.coli* involves five proteins represented by UDG, AP endonuclease IV, RecJ, DNA polymerase I and DNA ligase and utilizes single nucleotide gap filling mechanism (20,21). In higher eukaryotes also, the replacement of dUMP by dCMP occurs by generation of single nucleotide repair patches (22,23). However, to date, there are no reports on the participation of any ancillary proteins that may be playing a role in recruiting UDG to the site of its action.

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Highly inefficient excision of uracil from DNA hairpin loops suggested that melting of these structures may be needed for efficient repair (24). A candidate macromolecule for melting such structures could be the single stranded DNA binding protein whose involvement in nucleotide excision repair in human cell lines has been already shown (25).

MATERIALS AND METHODS

Purification of UDG and SSB

UDG was purified from an overexpressing clone of *E.coli* TG1 harbouring pTrc99CUng (U. Varshney, unpublished). Constitutive expression of SSB was achieved by transforming a recombinant plasmid, pTL119A.SSB containing *ssb* gene (Lohman *et al*., 26; provided by Dr K. Muniyappa) into an *ung*$^{-}$ strain of *E.coli* BW310 (27). Other details of UDG and SSB purification were, in principle, as reported earlier (10,26,28). SSB was stored in a buffer containing 0.5 M NaCl to avoid precipitation of the protein at high concentrations (26) and desalted by chromatography on Sephadex G-50 prior to its use.

Oligodeoxyribonucleotides (oligonucleotides)

These were obtained from Bioserve Biotechnologies, Laurel (USA) and Regional DNA synthesis Laboratory at University of Calgary, Calgary (Canada). Oligonucleotides were purified (24) and quantified on Beckman DU 600 spectrophotometer using the resident software and made up to a final concentration of 10 pmol/µl. A list of the oligonucleotides used in this study is given in Table 1.

Enzymes, radioisotopes and other reagents

Enzymes were purchased from New England Biolabs or US Biochemicals and the radioisotopes were from Amersham. Other chemicals were from Boehringer Mannheim, Sigma or Gibco-BRL.

Labelling of oligonucleotides

Oligonucleotides (10 pmol) were 5′ ^{32}P-end-labelled using 20 µCi [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase in 10 µl reaction volumes (29). U-loop and U-stem oligonucleotides were labelled by filling in the 3′ recessed ends with 20 µCi [α-^{32}P]dCTP (5000 Ci/mmol) in the presence of 250 µM dATP, dTTP and dGTP using Klenow fragment of DNA polymerase I (2 U) in 10 µl reactions (30). The radiolabelled oligonucleotides were purified by chromatography on Sephadex G-50 minicolumns. This procedure routinely resulted in labelling efficiency of ~10^6 c.p.m./pmol oligonucleotide. For K_m and V_{max} determinations, radiolabelled oligonucleotides were mixed with cold substrates such that total contribution from labelled counterpart was much less than 1%.

Electrophoretic mobility shift assays

Oligonucleotides (1 pmol, ~10^5 c.p.m.) were incubated with SSB tetramer (5 pmol) in 15 µl UDG buffer at 27°C for 10 min, electrophoresed in cold (4°C) on 8% polyacrylamide gels (30:0.5 acrylamide:bis-acrylamide) using 0.5× TBE buffer (30) for 1–2 h at 150 V and autoradiographed.

Excision of uracil in the presence of SSB

Standardization of conditions showed that the effect of SSB on uracil excision was independent of whether or not it was preincubated with the substrates or UDG. However, for the reactions in the presence of SSB, substrates were routinely preincubated with it for 10 min at 27°C prior to the addition of UDG.

Range finding experiments

UDG reactions with the various dilutions of enzyme in 15 µl reaction volumes were performed (24) using 1 pmol substrate in the presence or the absence of 5 pmol SSB tetramer.

K_m and V_{max} determination

Reactions (15 µl) containing varying amounts of substrates were carried out using appropriate dilutions of UDG. Data were analysed as described (24) and K_m and V_{max} values were determined from double reciprocal plots (31) using the grapher software. As UDG is inhibited by high salt concentrations, for the determinations of K_m and V_{max} values in the presence of SSB, it was freed of the salts present in the storage buffer, prior to its use, by chromatography on Sephadex G-50 and quantified (32). SSB tetramer was obtained at a concentration ~3 pmol/µl. Such concentrations are inadequate to provide saturating molar excess of SSB tetramer at high substrate concentration points needed to perform Michaelis–Menten type kinetics. Hence, the experiments were performed in the presence of the equimolar ratios of SSB tetramer to the substrates.

Table 1. List of the oligonucleotides

S. No.	Name	Size (nt)	Sequence	Remarks
1	SS-U3	24	d(ct**U**aagtgcaggcatgcaagagct)	Single stranded substrate, U at 3rd position
2	SS-U4	25	d(agc**U**catagtttacctgaagaatat)	Single stranded substrate, U at 4th position
3	SS-U9	24	d(ctcaagtg**U**aggcatgcaagagct)	Single stranded substrate, U at 9th position
4	Loop-U1	22	d(ctag**aggatcc U**ttt **ggatcct**)	U at 1st position of the tetraloop
5	Loop-U2	22	d(ctag**aggatcc** t**U**tt **ggatcct**)	U at 2nd position of the tetraloop
6	Loop-U3	22	d(ctag**aggatcc** tt**U**t **ggatcct**)	U at 3rd position of the tetraloop
7	Loop-U4	22	d(ctag**aggatcc** ttt**U** **ggatcct**)	U at 4th position of the tetraloop
8	U-hairpin	24	d(gcatgcctgcac t**U**aa gtgcaggc)	U in the loop
9	U-loop	32	d(cgatctagaggatcc t**U**tt ggatcctctagat)	U in 2nd position of the tetra loop
10	U-stem	32	d(cgatctagaggatcc tttt ggatcc**U**ctagat)	U in the stem

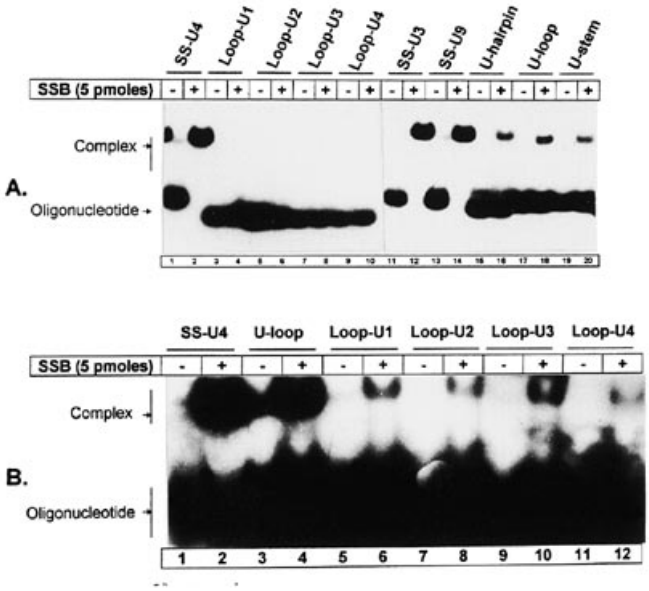

Figure 1. Electrophoretic mobility shift assays. (**A**) Oligonucleotides, 1 pmol (10^5 c.p.m.) were incubated with 5 pmol SSB (tetramer) in 15 µl UDG reaction buffer at 27°C for 10 min, electrophoresed on 8% polyacrylamide gel at 4°C for 2 h, and autoradiographed. (**B**) Same as (A) except that oligonucleotides were labelled to higher specific activity (1 pmol, 2.5×10^5 c.p.m.) and electrophoresed for 1 h. Presence (+) or absence (–) of SSB is indicated.

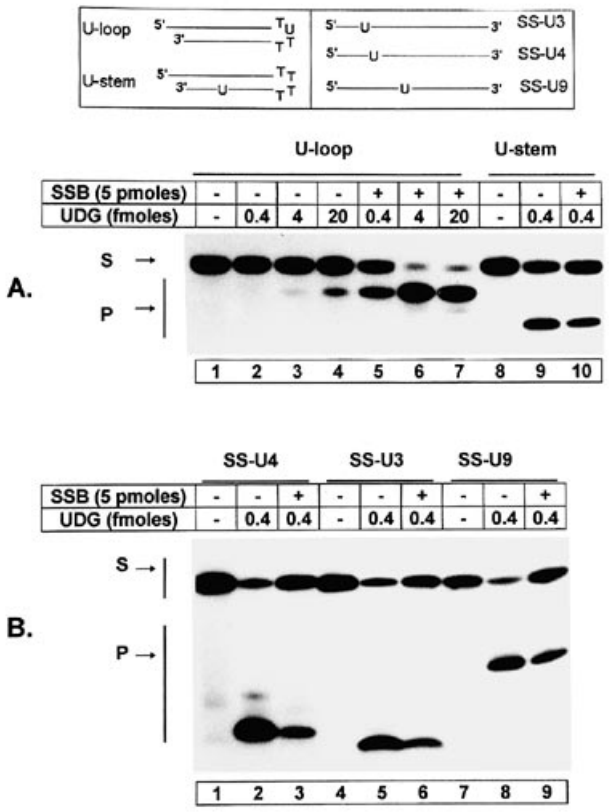

Figure 2. Effect of SSB on UDG reactions. Oligonucleotides (1 pmol) were either supplemented (+) with 5 pmol SSB (tetramer) or not supplemented (–) and the reactions carried out either in the absence (–) or the presence of the indicated amounts of UDG (0.4, 4 or 20 fmol). The samples were analyzed on 18% polyacrylamide, 8 M urea gels. The diagrammatic sketches of the various oligonucleotides are shown in the box.

Structural probing with $KMnO_4$

Oligonucleotides (~0.1 pmol, ~10^5 c.p.m.) were mixed with SSB tetramer (0.1–0.5 pmol) or 100 ng BSA in 10 µl UDG reaction buffer. To the mix, 2.5 µl freshly prepared 10 mM $KMnO_4$ was added and incubated at 27°C for 5 min. Reaction was terminated by addition of 7.5 µl β-mercaptoethanol (40 mM), 80 µl sodium acetate (0.375 M) and 50 µg/ml yeast total RNA. The oligonucleotides were ethanol precipitated twice, washed with 75% ethanol, suspended in 100 µl piperidine (1 M), and heated at 90°C for 30 min. The samples were frozen at –70°C, lyophilized, resuspended in 10 µl formamide loading dye and aliquots (5 µl) were analysed on 18% polyacrylamide–8 M urea gels of 0.4 mm thickness (33). The control samples were treated the same except that $KMnO_4$ was not added to the tubes.

RESULTS

Characterization of oligonucleotides

A list of oligonucleotides and the abbreviations used to denote them is given in Table 1. Treatment of radioactively labelled oligonucleotides with excess UDG resulted in essentially 100% excision of uracil from the expected positions of all substrates (data not shown).

Interaction of single stranded DNA binding protein (SSB) to oligonucleotides

To investigate the involvement of SSB, we analysed its ability to bind to different oligonucleotides by electrophoretic mobility shift assay (EMSA). As shown (Fig. 1A), in the presence of 5-fold molar excess of the SSB tetramer (34), SS-U3 (lanes 11 and 12), SS-U4 (lanes 1 and 2) and SS-U9 (lanes 13 and 14) form complex with SSB and no free oligonucleotides were detected. These oligonucleotides were mostly in complex with SSB even in the presence of an equimolar amount of SSB (data not shown). On the other hand, even at 5-fold molar excess of SSB tetramer only ~10% of U-hairpin (lanes 15 and 16), U-loop (lanes 17 and 18) and U-stem (lanes 19 and 20) were in the complexes, suggesting a weak interaction of SSB with these oligonucleotides. Under the same conditions, the oligonucleotides Loop-U1, -U2, -U3 and -U4 did not show any detectable complex (lanes 3–10). However, when we performed EMSA using the oligonucleotides labelled to higher specific activity and decreased the electrophoretic run time, complex formation could be detected with these substrates (Fig. 1B, lanes 5–12). Thus compared to SS-U4 (Fig. 1B, lanes 1 and 2) or to U-loop (Fig. 1B, lanes 3 and 4), complexes of Loop-U1, -U2, -U3 and -U4 with SSB are very weak.

Effect of SSB on uracil excision from U-loop, SS-U3, SS-U4 and SS-U9

To study the effect of SSB on uracil release, the substrates were incubated with 5-fold molar excess of SSB tetramer before UDG reactions were carried out (Fig. 2). In the absence of SSB, only at the highest concentration of UDG (20 fmol, Fig. 2A, lane 4), some product release is seen from U-loop. In the presence of SSB, even at the lowest concentration of UDG (0.4 fmol, lane 5) the product release is at least 3–4-fold higher than that from its

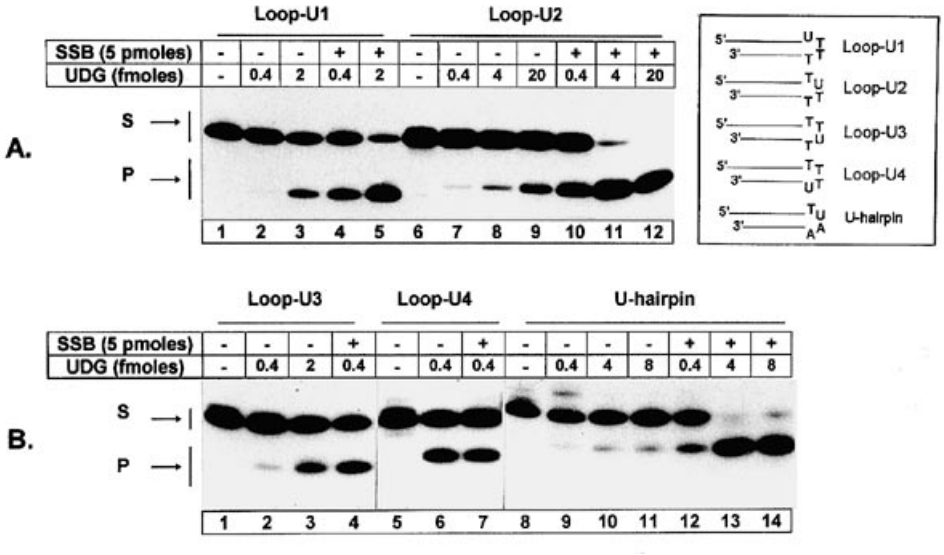

Figure 3. Effect of SSB on UDG reactions. Oligonucleotides (1 pmol) were either supplemented (+) with 5 pmol SSB (tetramer) or not supplemented (–) and the reactions carried out either in the absence (–) or the presence of the indicated amounts of UDG (0.4, 4 or 20 fmol). The samples were analysed on 18% polyacrylamide, 8 M urea gels. The diagrammatic sketches are shown in the box.

highest concentration (20 fmol) in the absence of SSB (compare lanes 4 with 5). Surprisingly, when we performed similar experiments on single stranded substrates, SS-U3, -U4 and -U9, the efficiency of uracil release was lowered by the presence of SSB (Fig. 2B). The three substrates used contained dUMP at the varying locations, and therefore, the effect of SSB was position independent. A similar but less pronounced effect of SSB was also seen on uracil release from a double stranded substrate, U-stem (Fig. 2A, lanes 8–10).

To further examine the effect of SSB on uracil release from loop regions, Loop-U1, -U2, -U3 and -U4 containing dUMP in either the first, second, third or the fourth position of the tetraloops were designed from the middle region of U-loop (Table 1). Another substrate, U-hairpin, whose sequence is altogether different from U-loop was used as a control to rule out sequence dependent effects. Excision of uracil from Loop-U1 (Fig. 3A, lanes 1–5), Loop-U2 (Fig. 3A, lanes 6–12), Loop-U3 (Fig. 3B, lanes 1–4) and U-hairpin (Fig. 3B, lanes 8–14) was poor in the absence of SSB but increased significantly in its presence. On the other hand, uracil release from Loop-U4 was efficient and remained mostly unchanged in the presence of SSB (Fig. 3B, compare lanes 6 with 7).

Kinetics of uracil release in the presence of SSB

To study the kinetics of uracil release as a function of SSB concentration, UDG reactions were carried out in the presence of the varying concentrations of SSB tetramer to the substrates (Fig. 4). It is clear that the single stranded ('unstructured') oligonucleotides SS-U3, -U4 and -U9, all of which are good substrates (~80% excision in the absence of SSB) show gradual decrease in the efficiency of uracil release with increasing SSB concentration up to the molar ratio of ~2.5. Subsequently, no further decrease is seen up to the molar ratios of 10 or 15 (Fig. 4, left panel). The data show that SSB results in 2–3-fold decrease in the efficiency of uracil release from single stranded substrates. In contrast, uracil release from loop regions gradually increases with increasing concentrations of SSB. At the substrate to SSB molar ratios of 2.5 to 5 a plateau is reached with no further changes up to a molar ratios of 10 (Fig. 4, right panel). The only exception to this analysis is Loop-U4, which showed a small but detectable decrease in uracil release. One of the substrates, SS-U4 was also preincubated with excess BSA (instead of SSB), as a control. We observed that increased concentration of protein *per se* did not influence the UDG activity (data not shown). An important conclusion from this data is that the presence of saturating levels of SSB tetramer (5-fold molar excess) resulted in near uniform release of uracil from all the substrates (~30–50%) which in its absence varied more than two orders of magnitude (see below, Tables 2 and 3). At the amounts of UDG used (0.4 fmol), release of uracil from the loop region of the hairpins (except for Loop-U4) is insignificant in the absence of SSB (re: Figs 2 and 3). Hence, the fold increase in the efficiency of uracil release from the hairpin substrates could not be accurately estimated from this experiment.

Figure 4 showed that the 5-fold molar excess of SSB tetramer to substrate represents a concentration beyond which the extent of uracil release does not change for any of the substrates. As the experiments shown in Figures 2 and 3 were performed in the presence of 5-fold molar excess of SSB tetramer, the data were quantified to estimate the increase or decrease in the efficiency of uracil release from the various substrates (Table 2). In the presence of SSB, the excision of uracil from the second position of tetraloops (U-loop, Loop-U2), and U-hairpin is improved 43–137-fold. The excision of uracil from the first and the third positions of the tetraloops is also increased ~7-fold. In contrast, the presence of SSB results in 2–3-fold decrease of uracil release from single stranded substrates, SS-U3, -U4 and -U9, and a double stranded substrate, U-stem (dUMP in the stem region). A slight decrease in uracil excision from Loop-U4 is also evident.

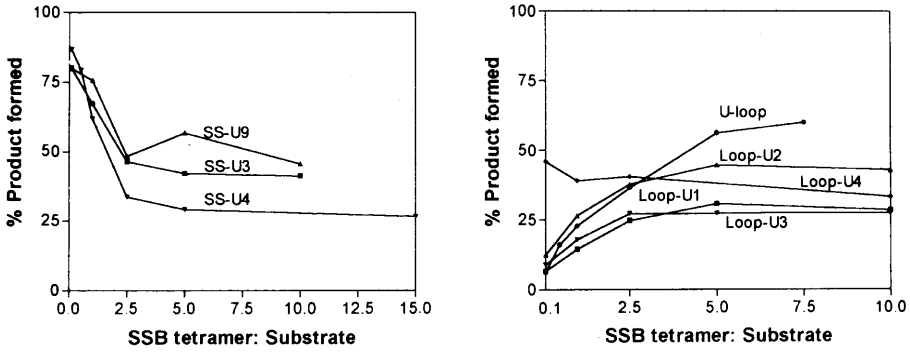

Figure 4. Release of uracil at different concentrations of SSB. Substrates (1 pmol) were incubated with the varying molar ratios of SSB (tetramer) for 10 min at 27°C and subjected to reaction with 0.4 fmol UDG. The reaction products were electrophoresed on 18% polyacrylamide, 8 M urea gels and autoradiographed. Radioactivity in the bands corresponding to the product and the remaining substrate was determined to calculate the percent product formed as 100[product/(left over substrate + product)].

Table 2. Effect of SSB on the excision of uracil

S. No.	Substrate	% Product formed (–SSB)	% Product formed (+SSB)	Ratio (+SSB/–SSB)
1	SS-U4	84	29	0.345
2	SS-U3	65	27	0.415
3	SS-U9	75	40	0.533
4	U-stem	52	29	0.55
5	U-loop	0.16	22	137.5
6	U-hairpin	0.13	5.6	43
7	Loop-U1	2.9	20	6.9
8	Loop-U2	0.18	15.5	86.1
9	Loop-U3	2.7	20.5	7.6
10	Loop-U4	37	30	0.81

Quantitation of the data shown in Figures 2 and 3. Bands corresponding to the product and the remaining substrate from the selected lanes were cut out, radioactivity counted and used to calculate percent product formed per 0.4 fmol UDG. Percent product formed was calculated as 100[product formed/(substrate left at the end of reaction + product formed)]. The data used corresponded to the various reactions as follows. SS-U4 (Fig. 2B, lanes 2 and 3). SS-U3 (Fig. 2B, lanes 5 and 6). SS-U9 (Fig. 2B, lanes 8 and 9). U-stem (Fig. 2A, lanes 9 and 10). U-loop (Fig. 2A, lanes 4 and 5). U-hairpin (Fig. 3B, lanes 11 and 12). Loop-U1 (Fig. 3A, lanes 3 and 4). Loop-U2 (Fig. 3A, lanes 9 and 10). Loop-U3 (Fig. 3B, lanes 3 and 4). Loop-U4 (Fig. 3B, lanes 6 and 7).

Kinetic parameters of uracil excision in the absence and presence of SSB

To further understand the mechanism of uracil release from the various substrates, the kinetic parameters (K_m and V_{max}) of uracil excision from various oligonucleotides were determined (Table 3). The efficiency of uracil excision was calculated as V_{max}/K_m ratio and represented as percent of the reference substrate SS-U4. Uracil release from the loop regions of DNA hairpins Loop-U1, Loop-U2 and Loop-U3 is inefficient (3.21%, 0.37% and 5.9%, respectively). Of these, Loop-U2 is the most inefficient substrate because of the combined effect of the high K_m and low V_{max} values. Interestingly, uracil excision from the fourth position of tetraloop is efficient (66.8%) and the kinetic parameters suggest that the favourable K_m of the enzyme for Loop-U4 largely contributes to making it a better substrate.

To understand the mechanism of SSB effect on uracil release, we also determined the K_m and V_{max} values in the presence of SSB using SS-U4, Loop-U2 and -U4 as representative substrates. Since uracil excision from Loop-U4 is not significantly affected by SSB, it served as a control. Due to technical reasons these studies were performed at substrate to SSB tetramer molar ratio of 1:1 (see Materials and Methods). The results (Table 3, lower half) show that the decreased rate of uracil excision from the single stranded substrate (SS-U4) is largely due to decrease in the V_{max} (214 versus 675) and the K_m values are not significantly affected (5.25 versus 6.57). On the other hand, better utilisation of Loop-U2 is a result of both increased V_{max} (408 versus 15.2) and decreased K_m (13.88 versus 39.9). Hence, the decrease in uracil excision from single stranded substrates is mostly due to the decreased catalytic rate whereas an enhanced uracil release from Loop-U2 is a combined effect of the increased catalytic rate and better substrate binding. As expected, in the case of Loop-U4 neither the K_m nor the V_{max} are altered significantly by the presence of SSB.

Structural probing of SS-U4 with $KMnO_4$

$KMnO_4$ preferentially attacks T residues in the single stranded regions of DNA (35) and has been widely used as a probe to detect sharply distorted or melted regions of DNA (36). We used $KMnO_4$ to detect SSB induced structural perturbations in the substrates. The results obtained with SS-U4 are shown in Figure 5. As expected, in a control where excess BSA (in place of SSB) was added to the reaction, we did not detect any alterations in the cleavage pattern of SS-U4 by $KMnO_4$ (compare lanes 4 with 5). Results of the various other controls shown in lanes 1, 2 and 3 are as expected. However, in the presence of SSB at 1:1 or 1:5 molar

ratios (lanes 6 and 7), some changes in the cleavage pattern are observed. The thymine residues (T16 and T23) showed enhanced cleavage. More importantly, the thymine residues (T7 and T10) in the vicinity of the dUMP residue showed decreased cleavage in the presence of SSB (compare lanes 6 and 7 with 4). Furthermore, the products arising as a result of the slow chemistry of $KMnO_4$ induced cleavage (marked by asterisk, corresponding to G9) showed decreased reactivity in the presence of SSB. These observations suggest that the presence of SSB results in decreased accessibility of the nucleotide bases (proximal to dUMP) to $KMnO_4$ in the single stranded substrate SS-U4. The enhanced cleavage at positions T16 and T23 is unclear at present.

Table 3. Kinetic parameters of uracil excision from the various substrates

S. No	Substrate	K_m[a] ($\times 10^{-7}$ M)	V_{max}[b] ($\times 10^2$)	Relative[c] V_{max}/K_m	Ratio[d] (+SSB/–SSB)
In the absence of SSB					
1	SS-U4	6.57	675.7	100	n. a.
2	Loop-U1	39.9	132	3.21	n. a.
3	Loop-U2	39.9	15.2	0.37	n. a.
4	Loop-U3	22.7	127.9	5.9	n. a.
5	Loop-U4	2.52	173.5	66.8	n. a.
In the presence of SSB					
6	SS-U4.SSB	5.25	214	39.6	0.396
7	Loop-U2.SSB	13.88	408.7	28.58	75.2
8	Loop-U4.SSB	2.45	153	60.7	0.908

n.a., not applicable.
All the values are average of two to four independent experiments.
[a]K_m values are for the uracil residue in the oligonucleotides.
[b]V_{max} values are in pmol product formed/min/μg protein.
[c]Relative V_{max}/K_m are shown as % of SS-U4.
[d]Ratio of V_{max}/K_m values (+SSB/–SSB).

Structural probing of Loop-U2 and U-hairpin with $KMnO_4$

The presence of SSB resulted in $KMnO_4$ induced cleavage at T9 and T19 positions (Fig. 6A, lanes 7 and 8). These thymines are involved in base pairing (3rd and 4th base pair from the loop closing base) and in the absence of SSB show no modification by $KMnO_4$ (lanes 5 and 6). In addition, the cleavage of thymine residues in the loop region (T12, T14 and T15) is also distinctly enhanced in the presence of SSB. Cleavage of the loop uracil, U13 (through a slow chemistry, marked by asterisk) also showed a definite increase in the presence of the SSB. Taken together, $KMnO_4$ probing suggests melting/opening of the hairpin structure in the presence of SSB. These structural perturbation may result in display of loop residues for improved recognition by UDG.

To further establish that SSB leads to destabilization of hairpin structure, structural mapping of yet another oligonucleotide, U-hairpin was perfomed (Fig. 6B). Similar to the findings with Loop-U2, presence of SSB resulted in $KMnO_4$ induced cleavage at positions T8 and T18 (Fig. 6B, compare lanes 6 and 7 with 4 and 5). This suggests melting of the double stranded structure in this region. In addition, cleavage at position T13 (just 5′ to the dUMP residue) was also enhanced in the presence of SSB. These findings reinforce the conclusion drawn above that the presence of SSB results in melting of the hairpin structures.

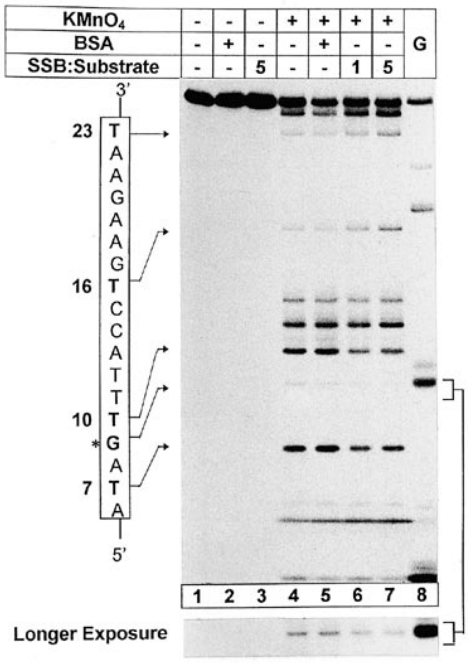

Figure 5. Structural probing of SS-U4 with $KMnO_4$. Reactions were performed as described in Materials and Methods. Lanes 1–3, oligonucleotide processed through all the steps but not treated with $KMnO_4$. Lane 1, neither BSA nor SSB were added; lane 2, 100 ng BSA was added; lane 3, SSB added at 5-fold molar excess. Lane 8, dimethyl sulfate generated G ladder. Lanes 4–7, $KMnO_4$ induced cleavage pattern. Lane 4, neither BSA nor SSB were added. Lane 5, 100 ng BSA was added. Lanes 6 and 7, SSB was added in equimolar or 5-fold molar excess to the oligonucleotide, respectively. Various positions sensitive to the reagent are shown by arrows.

DISCUSSION

We have used synthetic DNA substrates harbouring dUMP in different structural contexts to understand the mechanism of action of UDG. Demonstration of pd(UN)p as the minimum size substrate for UDG (37) has provided crucial biochemical evidence in establishing the role of the backbone phosphates flanking the uracil residue in making specific contacts with the enzyme (13). Furthermore, these substrates also serve as a model system for the complex DNA structures that may be encountered *in vivo*.

We earlier showed that compared with a single stranded reference substrate, uracil excision from the second position of a tetraloop was highly inefficient. However, the substrates used did not allow us to address the question of the efficiency of uracil excision from the other positions in the loop (24). The present set of substrates where each one of the dTMP residues of the tetra T loop was systematically replaced with dUMP, not only confirms our earlier observations but also shows poor excision of uracil from the first and the third positions. However, its excision from the fourth position is efficient. The kinetic studies suggest that Loop-U1, -U2 and -U3 bind to UDG, 10–20 times less efficiently than Loop-U4 (Table 3). Further, the V_{max} of UDG for Loop-U2 is ~10 times lower than that for Loop-U1, -U3 and -U4 and demonstrate that the inefficient cleavage of uracil from the second position is a combined effect of both high K_m and low V_{max} values. The low K_m for Loop-U4 raises an intriguing possibility that the conformation of the sugar–phosphate backbone/uracil

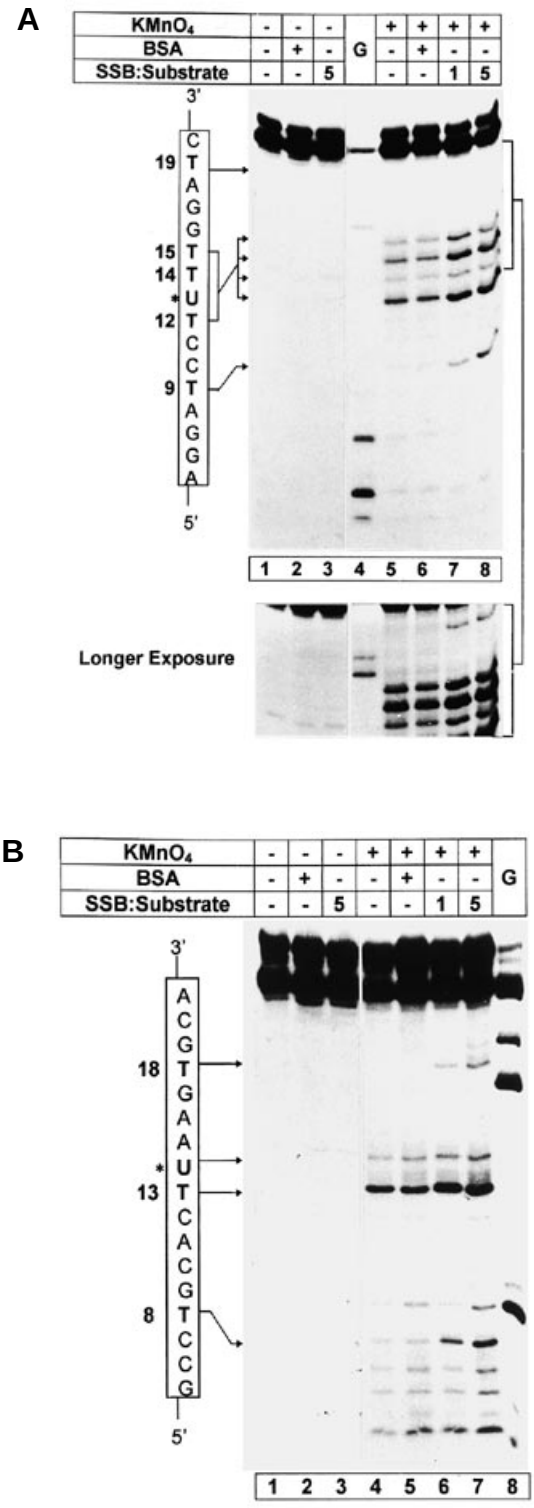

Figure 6. Structural probing of Loop-U2 (**A**) and U-hairpin (**B**) with $KMnO_4$. Reactions were performed as described in Materials and Methods. Lanes 1–3, same as in Figure 5. Dimethyl sulfate generated G ladder is shown in lane 4 (A) and lane 8 (B). Lanes 5–8 (A) and 4–7 (B) represent $KMnO_4$ induced cleavage pattern. Lanes 5 (A) and 4 (B), neither BSA nor SSB was added. Lanes 6 (A) and 5 (B), 100 ng BSA was added. Lanes 7, 8 (A) and 6, 7 (B), SSB added in equimolar or 5-fold molar excess as shown. The various positions sensitive to the reagent are indicated by arrows.

base of this substrate could be such that it docks favourably on to the active site groove of UDG (38). More interestingly, our present studies reveal the contrasting effects of SSB on excision of uracil from the various structural contexts. It decreases uracil release from single stranded ('unstructured') substrates by lowering the V_{max} whereas the uracil release from the loop regions is enhanced as a result of the slight decrease in K_m but greatly due to the remarkable increase in V_{max} (Table 3). Chemical probing using $KMnO_4$ suggests that SSB causes structural changes in the substrates resulting in decreased accessibility of the bases in the single stranded ('unstructured') oligonucleotides (Fig. 5) but increased accessibility in the hairpin substrates (Fig. 6A and B). The structural changes are thus consistent with the observed changes in the efficiencies of uracil release from the various substrates in the presence of SSB.

Uracil excision from the single stranded substrates is lowered 2–3-fold when annealed to the complementary DNA strand (10,24,39–41). A similar observation is made when the single stranded substrate forms a complex with SSB (Tables 2 and 3). Our results suggest that the mechanism of uracil recognition in the single stranded DNA–SSB complex may be similar to that in the double stranded substrates which has recently been shown to involve nucleotide flipping (13,17). In the single stranded DNA–SSB complex, the DNA wraps over the SSB tetramer (34) and a single stranded undecameric oligonucleotide has been shown to bind to SSB tetramer in a helical conformation of right handed B-type helix (42). Furthermore, as the DNA interacts with SSB through the nucleotide bases (34,42), the SSB tetramer may be considered a 'second strand DNA mimic'. Similar to the loss of the accessibility of thymines to $KMnO_4$ upon base pairing with the complementary strand DNA, the presence of SSB also results in decreased accessibility of thymines to the reagent (Fig. 5). Such an interaction, as is the case in double stranded DNA, may utilize the elements of recognition that are also needed for uracil binding in the active site pocket of UDG. For instance, in the single stranded DNA–SSB complex, the purine and pyrimidine bases are involved in stacking interactions against tryptophan residues of SSB (34). Similarly, one of the interactions that uracil makes in the active site pocket of UDG is by stacking against phenylalanine (11,12). This, in essence, makes some form of uracil base flipping or destabilization of the uracil and SSB contacts, a precondition for recognition by UDG. Since much of the decrease in the efficiency of uracil release from single stranded substrates in the presence of SSB is a result of lowered V_{max}, the proposed base flipping from the single stranded DNA–SSB complex may be the rate limiting step causing 2–3-fold poor utilization of the single stranded substrate (Table 2).

On the other hand, the hairpin substrates with uracil in position 1, 2 or 3 of the tetraloops are very poor substrates (0.3–5.5% as efficient as the reference). A likely reason for this is the interactions of the bases within the loop and/or the conformation of the sugar–phosphate backbone (38,43) which result in abstraction and binding of uracil into the active site pocket of UDG less favourable. In the presence of SSB, the hairpin structures are melted (Fig. 6A and B) and utilized efficiently by UDG (Table 3). A caveat to this interpretation, however, is that in the electrophoretic mobility shift assays, we detected at the best ~5–10% of the hairpin substrates into complex with SSB (Fig. 1). How does such a low level of substrate–SSB complex result in an increase of uracil excision up to two orders of magnitude (re: U-loop or Loop-U2, Table 2)? An argument that during the course of

enzyme reaction, more of the substrate is driven into a complex with the SSB tetramer is unlikely because the product of UDG reaction (an abasic site at the position occupied by uracil but with intact sugar–phosphate backbone) would still be expected to remain bound to SSB and therefore no depletion of the substrate–SSB complex. From another perspective, if only ~5–10% of the substrate is in complex with SSB, distinct cleavage enhancements by $KMnO_4$ (Fig. 6A and B) are not expected over the high background. Hence, the results of $KMnO_4$ probing, in turn, suggest an intermediary or transient state of DNA–SSB complex, which is not detectable by EMSA but exists in solution. We believe that it is this state of substrate–SSB complex which largely contributes to the enhanced excision of uracil by UDG. Furthermore, the relative ease of the ends of the stem to 'breathe', may facilitate nucleation of SSB on hairpin substrates and result in the destabilization of the interactions in the loop region. As uracil excision from the double stranded substrates is better than its release from the single stranded DNA–SSB complex (Table 2), decreased uracil excision from U-stem in the presence of SSB (Fig. 2A, lanes 9 and 10; Table 2) supports the view that SSB binding occurs from the stem region. This may also explain why the loop-U4 which is a good substrate to begin with does not show much effect of SSB on uracil excision by UDG.

Finally, our present findings are also important for various applications of UDG in molecular biology (44–50). The use of UDG as a uracil specific modifying agent had long been suggested in DNA sequence analysis related applications (1), however its use for this purpose has not become popular mostly due to the wide range of efficiency (0.37–100%) with which uracil is excised from DNA polymers (24,41,51–53). The results shown in Figure 4, clearly suggest that in the presence of SSB, uracil is excised with a fairly uniform efficiency (30–50%) from different structural contexts. Thus, our studies should promote the use of UDG in DNA sequence analysis related applications.

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