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# Correlated cleavage of single- and double-stranded substrates by uracil-DNA glycosylase

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**Abstract** **Uracil-DNA glycosylase (Ung) can quickly locate uracil bases in an excess of undamaged DNA. DNA glycosylases may use diffusion along DNA to facilitate lesion search, resulting in processivity, the ability of glycosylases to excise closely spaced lesions without dissociating from DNA. We propose a new assay for correlated cleavage and analyze the processivity of Ung. Ung conducted correlated cleavage on double- and single-stranded substrates; the correlation declined with increasing salt concentration. Proteins in cell extracts also decreased Ung processivity. The correlated cleavage was reduced by nicks in DNA, suggesting the intact phosphodiester backbone is important for Ung processivity.**


*Keywords:* DNA repair; Base excision; Processivity; Correlated cleavage; Uracil-DNA glycosylase

## 1. Introduction

DNA glycosylases excise damaged bases from DNA, initiating a process of base excision repair [1]. Finding sparse lesions in DNA is a formidable challenge; therefore, DNA glycosylases must unavoidably bind DNA non-specifically and then travel somehow to their cognate lesions. Kinetic and thermodynamic analysis of possible search mechanisms suggests that three-dimensional diffusion would be inefficient [2,3]. A possible solution is its supplementation with one-dimensional diffusion of the searching protein along DNA (“sliding”) in combination with dissociation of the protein from DNA and immediate reassociation to the same DNA segment nearby (“hopping”) ([2,4]). Sliding and hopping are “correlated search” modes, in which consequently sampled DNA stretches are strongly correlated in one-dimensional space. Correlated search was advocated for many proteins recognizing specific DNA elements, such as restriction endonucleases, transcription regulators, repair enzymes, etc. [4]. However, it is unclear whether sliding can successfully operate in cellular environment, with its high ionic strength (disfavoring one-dimensional diffusion by screening out electrostatic protein interactions with non-specific DNA [2]) and many other DNA-binding molecules.

Several DNA glycosylases are suggested to use the one-dimensional search, inferred from the phenomenon of “processivity”, the ability of glycosylases to excise several lesions separated by a small distance (~10–25 nt) without releasing the DNA substrate [5–11]. Processive cleavage implies that the enzyme uses correlated search over the distances comparable to the mean separation distance between the lesions [4]. Opposed to the processive cleavage is a distributive one, with the enzyme releasing DNA after each catalytic act and binding DNA again in an uncorrelated place. In full accordance with theoretical predictions for one-dimensional search, DNA glycosylases switch from processive to distributive mode with increasing salt concentrations [6,7,9–11].

Studies of processivity of DNA glycosylases are currently limited by two available assays. In the plasmid cleavage assay, a plasmid is randomly damaged, and the rates of conversion of supercoiled DNA to its relaxed and linear form by DNA glycosylases are compared. Processivity is revealed as rapid appearance of the linear form due to fast cleavage of closely opposed lesions [5,9]. In the concatemeric substrate assay, a long DNA consisting of identical repeated lesion-containing oligonucleotide (ODN) units is constructed by ligation, and the rates of accumulation of monomeric units and products of other lengths are compared [11,12]; the processive action produces mostly monomers. These assays lack the ability to control (plasmid assay) or manipulate (both assays) the substrate structure, precluding characterization of the mechanisms of correlated search. For instance, using the concatemeric substrate assay with differently constructed substrates, uracil-DNA glycosylase (Ung) from *Escherichia coli* was reported to be either processive [10] or distributive [12].

We report a new quantitative ODN-based assay, which relies on the correlated cleavage to assess processivity of DNA glycosylases or other enzymes recognizing DNA modifications or sequences, and permits full control over the substrate structure. Using this assay, we analyze the correlated excision of Ura by purified Ung and in cell-free extracts, including the first analysis of Ung processivity on its preferred ssDNA substrates. Modifications of the substrate clarify the relationship between sliding and hopping during the correlated cleavage.

## 2. Materials and methods

### 2.1. Enzymes and oligonucleotides

Ung, T4 polynucleotide kinase, and T4 DNA ligase were from New England Biolabs. ODNs were synthesized from phosphoramidite precursors using established protocols. The modified 20-mer 3′-ODN, d(GGACTTCUCTCCTTTCCAGA) (600 pmol), was 5′-labeled using $\gamma[^{32}P]$ ATP and polynucleotide kinase (10 U, 37 °C, 40 min, then another 10 U of kinase added for 40 min). In a separate reaction, the

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same ODN was phosphorylated using 1 mM non-radioactive ATP in the same way. After 10-min heating at 95 °C, both reaction mixtures were pooled, combined with a 1.5-fold excess of the 20-mer 5′-ODN, d(TCCCTTCUCTCCTTTCCTTC), and the 40-mer complementary strand d(TCTGGAAAGGAGCGAAGTCCGAAGGAAAGGAGCGAAGGGA), and annealed as follows: 1 min 95 °C, 10 min 57 °C, 2 h gradual cooling to 25 °C. This regime minimized unproductive annealing (5′-ODN → 5′-ODN, 3′-ODN → 3′-ODN, 3′-ODN → 5′-ODN). To the annealed product, ATP (1 mM final) and DNA ligase (3 Weiss units) were added and the mixture was left overnight at 4 °C. The ligated radioactive 40-mer was purified by denaturing PAGE and, if necessary, annealed to the 1.5-fold excess of the 40-mer complementary strand or two separate 20-mer complementary strands, d(TCTGGAAAGGAGCGAAGTCC) and d(GAAGGAAAGGAGCGAAGGGA).

### 2.2. Cell extracts

LB medium (5 ml) was inoculated from a frozen stock of *E. coli* DH5α. The culture was grown overnight at 37 °C and used to inoculate 360 ml of LB; the growth was continued until $A_{600} = 0.6$. The bacteria were collected by centrifugation (4 °C, 15 min, 12000 × *g*), resuspended in 20 ml of ice-cold 10 mM Tris–HCl (pH 8.0) and centrifuged again. The pellet was resuspended in a buffer containing 25 mM Na phosphate (pH 7.5), 5 mM ethylenediamine tetraacetate, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride (~4 ml final volume). The lysate was prepared by sonication (UZDN-2T dispergator, SELMI, Ukraine; six 15-s pulses at the highest power intermittent with 1.5 min on ice), clarified by centrifugation (4 °C, 20 min, 20000 × *g*), and stored at −70 °C.

### 2.3. Correlated cleavage assay

The reaction mixture contained 50 nM ODN substrate, 25 mM Na phosphate (pH 7.5), and 1 mM dithiothreitol. The reaction was initiated by adding Ung (final concentration, 0.013 U/ml for dsODN, 0.0077 U/ml for ssODN), or cell extracts (to 4 μg/ml total protein), incubated at 37 °C, and aliquots were withdrawn at 0.5, 1, 1.5, 2, 3, 5, 7, and 10 min. They were immediately quenched with NaOH (100 mM final), heated for 2 min at 95 °C, and neutralized with HCl. The products were resolved by denaturing PAGE and quantified with a Molecular Imager FX (Bio-Rad). Initial rates were determined from the linear parts of the time courses. If necessary, the reaction mixture was supplemented with 10, 25, 50, 100 or 200 mM KCl or with 500 nM ODN d(CTCTCCCTTCXCTCCTTTCCTCT) (X, a tetrahydrofuran abasic site) annealed to d(AGAGGAAAGGAGGGAAGGGAGAG).

## 3. Results and discussion

To analyze correlated cleavage of substrates by Ung, we have designed an ODN substrate containing two Ura residues and a radiolabel between them. The following consideration guided the design. First, single- and double-cleavage events must yield easily separated products. Second, the sequences around each Ura must be identical within the Ung footprint [13] to ensure the same cleavage efficiency at both sites. Third, the terminal sequences of the Ura-containing ODNs must allow unambiguous directional assembly of the ligated construct. The general structure of the substrate (S) and its sequence are shown in Fig. 1A. Cleavage at a single Ura residue produces radiolabeled fragments 32 or 27 nt long ($P_{32}$ and $P_{27}$, respectively), while cleavage at both Ura residues yields a 19-nt radiolabeled product ($P_{19}$).

As Ung is monomeric [14], under steady-state conditions ($[E]_0 << [S]_0$, $[P] << [S]_0$) there is a very low probability of two Ung molecules binding the same ODN, or of singly-nicked released product re-binding an enzyme molecule at the second Ura. Binding of one Ung molecule to the substrate followed by excision of one Ura base and ES complex dissociation can be

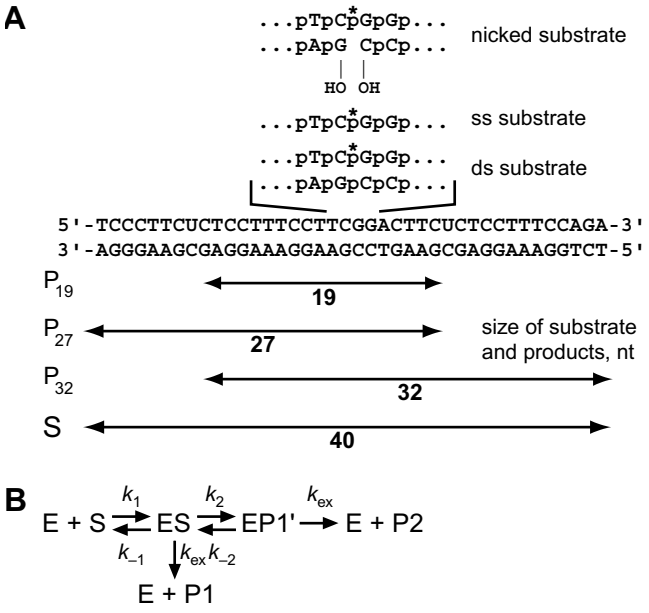


Fig. 1. General scheme of the experiment. (A) Structure and lengths of the DNA substrate and products. Asterisk indicates the $^{32}P$ phosphate. (B) Kinetic scheme of correlated cleavage.

described by a regular Michaelis–Menten scheme (characterized by constants $k_1$, $k_{-1}$ and $k_{ex}$ in Fig. 1B) and gives rise to either $P_{32}$ or $P_{27}$. Alternatively, the enzyme, after excising one Ura, could translocate to the other Ura residue in the same molecule (the EP1′ kinetic intermediate) and excise it ($k_2$, $k_{-2}$ and $k_{ex}$ in Fig. 1B, product $P_{19}$; note that the excision rate constant $k_{ex}$ is the same in both cases because, by design, the DNA sequences interacting with the enzyme are identical). The reaction kinetic graph [15] shows that the ratio of $P_{19}$ accumulation initial rate to the total cleavage rate can be presented as $k_2/(k_{ex} + k_2 + k_{-2})$. Making a reasonable assumption that $k_{-2} \ll k_2$ (i.e., once the enzyme has located the second Ura it excises this base instead of returning back to the first excision site), this can be simplified to $k_2/(k_{ex} + k_2)$. This equation gives "probability of correlated cleavage" ($P_{cc}$), the probability that the enzyme will proceed to make the second cleavage in the same substrate molecule. $P_{cc}$ provides a direct quantitative measure to compare efficiencies of correlated cleavage of a substrate under different conditions, or of different substrates with the same distance between the damaged bases. $P_{cc}$ is similar to the monomer/dimer ratio used to quantify correlation in concatemeric DNA assays [10,11,16], but is not troubled with omission of products larger than dimers, and can be converted to microscopic probabilities of dissociation and translocation assuming a random walk model [4].

A typical experiment to determine the initial rates of accumulation of $P_{32}$, $P_{27}$, and $P_{19}$ is shown in Fig. 2. $P_{32}$ and $P_{27}$ accumulated nearly linearly until 5–7 min, after which the rate of their increase started to decline, likely due to the ongoing conversion to $P_{19}$. The level of $P_{19}$ rose linearly over 10 min. The amounts of $P_{32}$ and $P_{27}$ were always nearly equal (Fig. 2A, marked by arrows), confirming similar efficiencies of cleavage at both Ura sites. Overall, even at 0 mM KCl the rate of correlated cleavage was below the rate of overall cleavage; the value of $P_{cc}$ extracted from the experiment shown in Fig. 2 was 0.41.

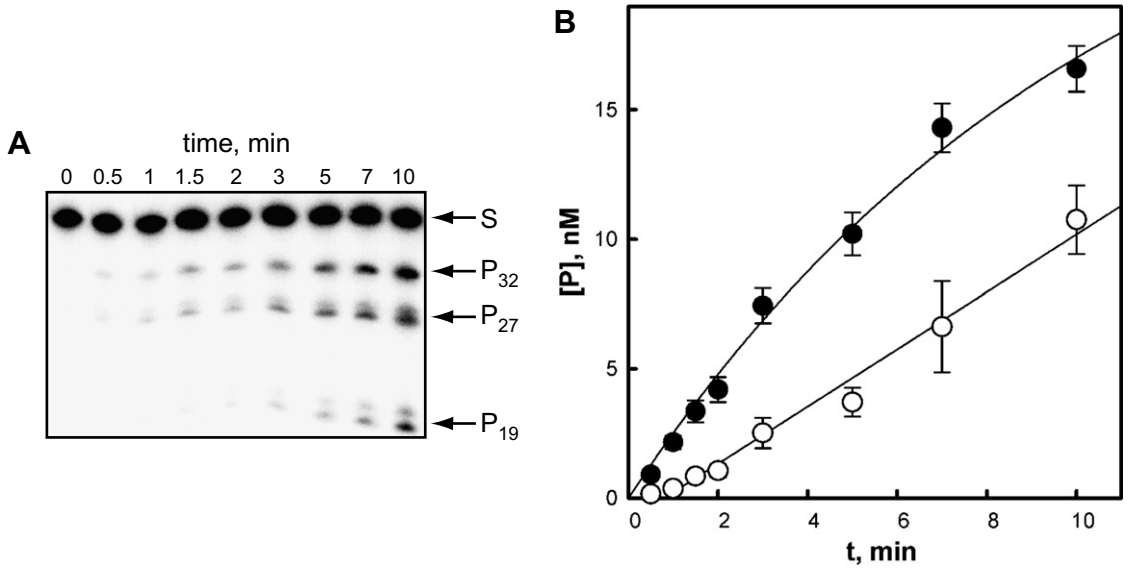


Fig. 2. Time course of substrate cleavage by purified Ung. (A) Ung cleaving dsODN at 0 mM KCl. No enzyme was added at the 0 min time point. Arrows indicate positions of the 40-mer substrate and products of different lengths after alkaline degradation of abasic sites produced by Ung. (B) Time course of accumulation of $P_{19}$ (○) and $P_{32} + P_{27}$ (●; shown as a sum to avoid overlapping). Mean ± S.D. of four independent experiments are shown.

In order to see whether $P_{cc}$ reflects the negative salt dependence expected for a diffusion-driven translocation process, we have determined $P_{cc}$ values for Ung cleaving the dsODN substrate at 0–200 mM KCl (Fig. 3, open symbols). $P_{cc}$ indeed decreased with increasing salt concentration, with no double cleavage observed at 200 mM KCl. The rates of accumulation of all products were reduced at higher salt concentration; however, the rate of $P_{19}$ accumulation declined faster, making overall $P_{cc}$ decrease. Therefore, $P_{cc}$ displayed the salt-dependence behavior mirroring that observed for Ung using plasmid cleavage [9] and concatemeric substrate assay [10].

Ung can utilize both double- and single-stranded substrates, preferring the latter [10,14]; processivity of Ung on ssDNA has not been studied before. We have analyzed correlated cleavage of a ssODN substrate of the sequence identical to the ds substrate, and examined salt dependence of $P_{cc}$ (Fig. 3). The enzyme was indeed more active on the ssODN, with less Ung required to achieve the same cleavage rate. However, $P_{cc}$ values for the ssODN were lower than for the dsODN at the same KCl concentrations, and dropped to nearly zero already at 100 mM KCl. This indicates that with ssDNA, the probability of dissociation after the first cleavage increases at the expense of the probability of translocation.

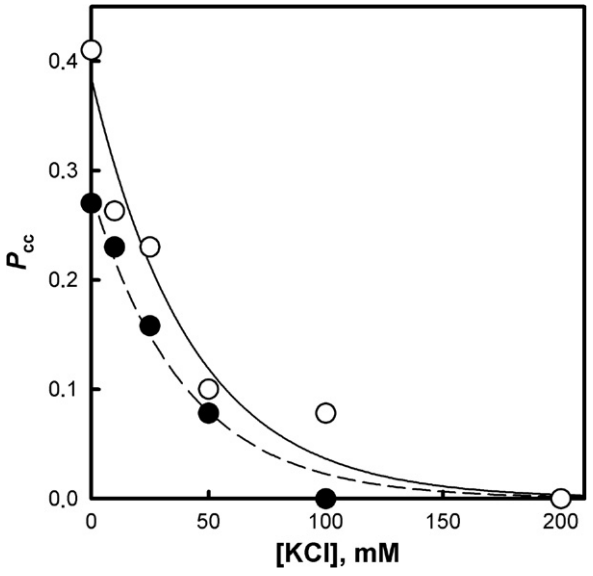


Fig. 3. Salt dependence of the correlated cleavage by Ung for dsODN (○) and ssODN (●) substrates.

Next we inquired whether correlated cleavage could be observed in bacterial cell extracts containing many DNA-binding proteins in addition to Ung. In addition to Ung, *E. coli* possesses another uracil-removing enzyme, Mug, which is strictly specific for Ura:Gua pairs [17,18]. As our substrate contains Ura:Cyt pairs, Mug should not contribute to its cleavage. At 0 mM KCl, the correlated cleavage of both ssODN and dsODN substrates was still observed, but its efficiency was significantly lower than for purified Ung (Fig. 4). $P_{cc}$ was 0.11 for ssODN and 0.12 for dsODN, suggesting that translocation along dsDNA may be more sensitive to interference from competing proteins. The overall efficiency of ssODN cleavage was lower than for the dsODN substrate, possibly due to better binding of ssODN by cellular proteins. Increased salt concentrations caused nearly a complete disappearance of $P_{19}$ (not shown).

To analyze the nature of translocation (sliding or hopping), a nick removing one phosphate was introduced in the middle of the non-modified strand of the dsODN. Such modification is expected to interfere with sliding without affecting hopping. With pure Ung, $P_{cc}$ for this substrate at 0 mM KCl was 0.31, however, the nick did not fully abolish correlated cleavage. The lower estimate of the contribution from sliding can be drawn from the assumption that the nick completely interrupts sliding; the 0.10 decrease in $P_{cc}$ of 0.41 means that at least ~25% of translocation at this particular ODN substrate is by sliding. To estimate the contribution from hopping, we have supplemented the reaction mixture with a competitor ODN

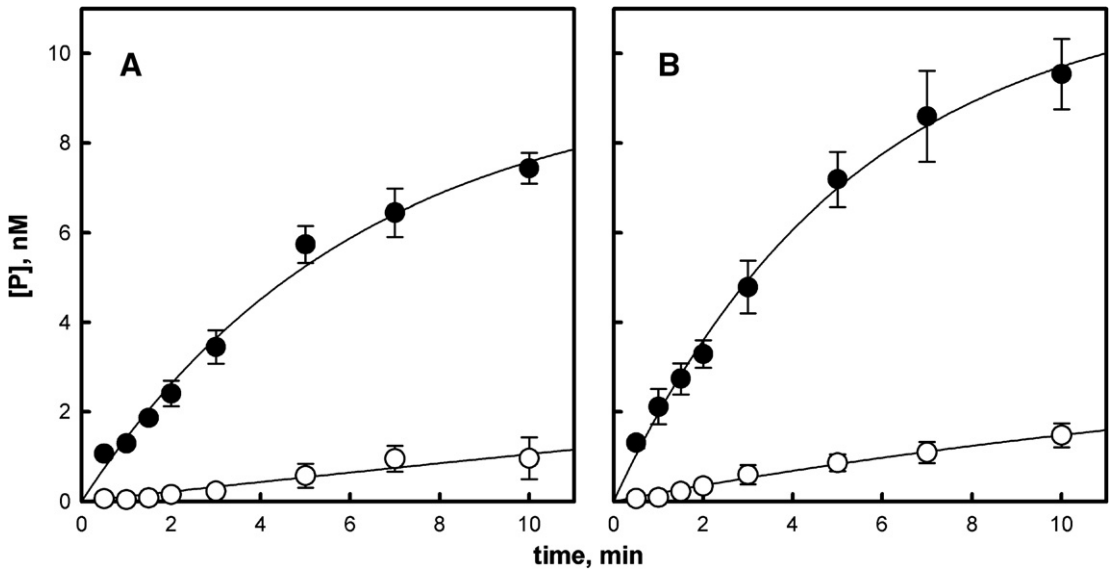


Fig. 4. Time course of accumulation of products of different lengths in reaction with *E. coli* cell extracts. Time course of accumulation of $P_{19}$ (○) and $P_{32} + P_{27}$ (●) during cleavage of ssODN (A) or dsODN substrate (B) at 0 mM KCl. Mean ± S.D. of three independent experiments are shown.

containing a tetrahydrofuran moiety, a good ligand for Ung [19]. When the competitor was introduced before Ung, 10 or 30 s after Ung, $P_{cc}$ decreased to 0.25–0.27, indicating that hopping participates in at least ~35% of translocation events. From these lower estimates, sliding and hopping contribute similarly (25–65% and 35–75%, respectively) to the overall translocation during correlated cleavage by Ung.

As the presence of salt and other proteins decreases the translocation of Ung, is facilitated diffusion as a search mechanism relevant in vivo? No data for Ung is presently available; however, in other cases a correlation between processivity in vitro and function in vivo has been shown. Accumulation of nicked plasmids follows the processive mode during repair in UV-irradiated *E. coli* cells by T4 endonuclease V [20]. Site-directed mutants of EcoRV, a restriction endonuclease that acts processively, demonstrate a correlation between processivity and the ability to protect *E. coli* from phage infection [21]. Thus, it cannot be excluded that correlated lesion search by Ung may operate in cells. The ODN-based assay we propose offers a convenient way into systematic investigation of the effect of substrate structure and reaction conditions on correlated cleavage by DNA glycosylases. Distance and phasing of the lesions can be varied, and the stretch of DNA between them can be modified to analyze the path of translocation and the diffusion constant. The same substrate design may be applied to other DNA-dependent enzymes.

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