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“No Strain, No Gain:” Studies in the Mechanism of a DNA Repair Enzyme*

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The presence of uracil in DNA occurs either as a result of the mis-incorporation of dUTP in place of dTTP or by deamination of deoxycytidine to give deoxyuridine and is pro-mutagenic. Some 500 such lesions are repaired per cell per day in man. The first enzyme in the repair pathway is uracil DNA glycohydrolase, UDG, which cleaves the glycosylic bond in deoxyuridine in DNA. It shows a rate acceleration of 10^{12} and specificity for uracil of at least 10^7 with respect to cytosine or thymine bases. Its mechanism of action has been revealed through the X-ray crystal structure of a transition-state analogue bound in the enzyme active site and is clearly a dissociative, S_N1 type process.

Key words: DNA repair, UDG mechanism, pseudo-deoxyuridine, enzyme:transition state analogue complex, enzymatic oxocarbenium ion

REPAIR of DNA is without question one of the most important processes in living organisms. The cellular DNA integrity is undermined by the damaging effects of numerous chemical and physical agents and any residual DNA damage interferes with primary DNA functions, especially transcription and replication, and so leads on to mutations or cell death. DNA damage arises from both endogenous sources such as water and oxygen and exogenous sources such as sunlight and tobacco smoke [1]. Faithful maintenance of the genome is crucial both to the individual and to the species and mechanisms restoring damaged DNA must necessarily have evolved early in evolution. They include [2] nucleotide and base excision repair [3], base-mismatch repair [4], base-deletion and base-insertion repair [5], and repair of single-strand and double-strand breaks [6]. In some cases, DNA damage is not repaired but is instead bypassed by specialized DNA polymerases [7].

In human cells, base alterations are generally removed by excision repair pathways that counteract the mutagenic effects of DNA lesions. This serves to maintain the integrity of the genetic information, although not all of the pathways are absolutely error-free. Recent studies of the molecular mechanisms of various DNA repair pathways suggest significant overlaps in their functions and direct interactions between DNA repair and DNA replication, transcription, cell cycle control, and

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* Dedicated to Prof. Jan Michalski on the occasion of his 80th birthday.
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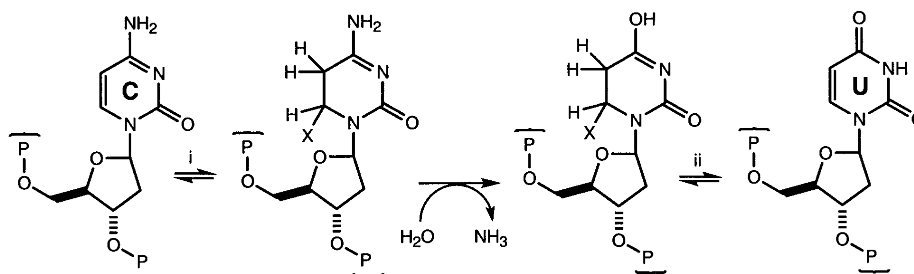
apoptosis have been observed. DNA repair must be both rapid and accurate. Clearly, it has to take place before DNA replication if cellular damage is to be avoided.

A base-excision repair pathway is an organism's primary defence against mutations induced by oxidative, alkylating, and other DNA-damaging agents that lead to modified nucleotide bases. This pathway is initiated by DNA glycosylases that excise the damaged base by cleaving the glycosylic bond between the base and the DNA sugar-phosphate backbone. A subset of glycosylases has an associated apurinic/apyrimidinic (AP) lyase activity that further operates on the abasic locus to cause single-strand cleavage of the DNA phosphate backbone. Chemical mechanisms that are supported by biochemical and structural data have been proposed for some glycosylases and glycosylase/AP lyases [8].

The most prevalent of these lesions is the conversion of deoxycytidine residues in DNA into deoxyuridines. That involves simply the deamination of the exocyclic amino group of the cytosine base, which has been recognised over many years as resulting from one of two chemical processes at neutral pH. The first involves the direct attack of water or hydroxide at C-4 of the cytosine ring. The second requires a reversible addition to the C5–C6 double bond of cytosine to enable spontaneous deamination of the intermediate 5,6-dihydrocytosine species. The rate constants for these combined processes have been determined [9] at elevated pH and temperature and extrapolated to 37°C to give $k_{hydrol} = 2 \times 10^{-10}\ s^{-1}$. Such saturation of the 5,6-double bond has been identified [10] to result from the formation of cytosine photohydrate or photodimer and also from the addition of mutagens, typically bisulfite [11] or hydroxylamine [12] at C6 of the cytosine ring (Scheme 1). In addition to deamination events in intact DNA, deoxyuridine residues in DNA can arise from mis-incorporation of dU (in place of dT) from deoxyuridine 5′-triphosphate, in spite of the existence of enzymes designed to rectify such events [13].

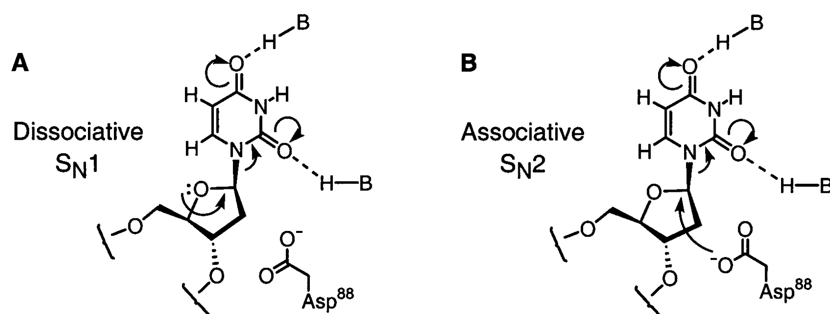


Scheme 1. Deamination of deoxycytidine residues to generate deoxyuridine residues in DNA. Reagents and conditions: i, HX (HX = HSO_3^-; H_2O/hν; or $HONH_2$); ii, loss of HX.

As a result of the combination of all of these possibilities for the deamination of cytosine residues in DNA, it has been estimated that there has to be enzymatic repair of between 100 and 500 deoxyuridine mismatches per cell per day in man [14] from a total of 10,000 damaged bases repaired per day [15]. The uracil-DNA glycosylases are a ubiquitous, highly conserved, and extremely specific class of DNA repair enzyme

[16]. They operate on single-stranded DNA rather faster than on double-stranded DNA with high efficiency for both dU:dA and dU:dG base-pairs by cleavage of the glycosylic bond from uracil to the deoxyribofuranose ring. Repair of dsDNA is completed by cleavage of the DNA backbone, removal of the resulting 5′-phosphate group, and the action of DNA polymerase and DNA ligase to replace the dU by dT [16]. The uracil DNA glycosylase is an archetypal human DNA base-excision repair (BER) enzyme that carries out the essential first step and has also been isolated from viruses and bacteria. Early protein crystal structures indicated that the enzyme binds an extrahelical uracil, thus providing an example of Roberts' base-flipping as a general mechanism for enzymes that carry out chemistry on deoxynucleic acid bases which are inaccessible when stacked inside the DNA helix [17].

Studies on the mechanism of action of this vital enzyme have been attempted by direct X-ray crystallographic analysis of viral [18], bacterial [19], and human [20] enzymes. However, the information derived from product complexes [20] or from complexes with a uracil base [19] were not able to reveal the catalytic mechanism. Extensive studies by spectroscopic methods, principally NMR [21] and Raman [22] spectroscopy, also gave only imprecise understanding of the mode of cleavage of the glycosylic linkage in dU. Several proposed mechanisms are inconsistent with each other and with the results of a molecular dynamics approach to the mechanism of glycoside cleavage, based on modelling from a product complex [23]. The majority of these proposals rely entirely on functional group chemistry and thus do not adequately explain the efficiency of UDG or the activity of mutants that alter evidently key functional groups [24,25]. While the amino acid residues in the uracil-binding site essential for catalysis could be identified and their state of ionisation examined, notable Asp-88 and His-210 (HSV numbering), the similarity of use of these residues for the alternative dissociative [25] (S_N1-like, Scheme 2A) and associative [26] (S_N2-like, Scheme 2B) processes made a distinction between these formal mechanisms virtually impossible (Scheme 2). Thus, knowledge of a substrate complex structure is essential to solve the mechanism of the UDG repair enzyme, especially since the co-crystal structure of a catalytically impaired UDG Asp145 $\rightarrow$ Asn mutant with deoxyuridine-containing DNA failed [25] to capture the uncleaved substrate as



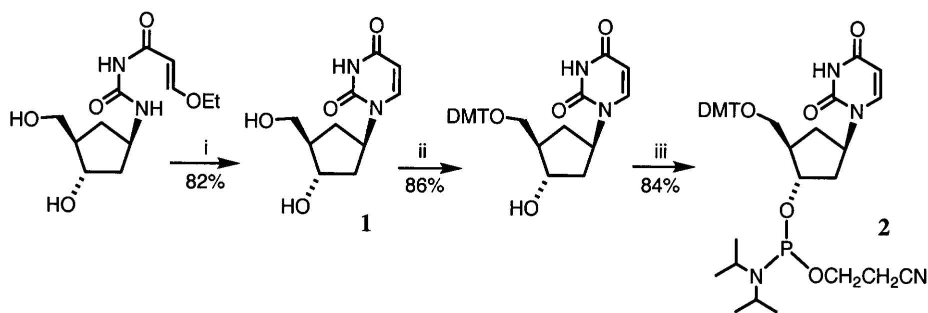
Scheme 2. Mechanistic alternatives for the cleavage of the glycosylic bond in deoxyuridine.

even the diminished enzyme maintained significant residual activity. This clearly indicates that functional group chemistry is not the primary basis for UDG catalysis.

Mechanistic studies on other enzymes that catalyse glycosyl transfer reactions [27,28] would suggest an S_N1-like, dissociative mechanism for the hydrolysis process for deoxyuridine [26], in which hydrogen bonding to the uracil base at the C^2 and C^4-carbonyl oxygens of the pyrimidine is coupled to dissociation of the C1′–N glycosylic bond supported by the anomeric effect [28]. This would lead to an oxocarbenium-ion with cationic charge stabilised between C-1′ and O-4′ of the deoxyribose residue and possibly further stabilised by coulombic interaction with the proximate Asp-88 anion or solvation in the active site (Scheme 2A). The alternative, associative process (Scheme 2B) invokes the nucleophilic participation of an oxyanion, either the essential Asp-88 or a water molecule activated by that residue [18].

We therefore initiated our studies by the design and synthesis of a stable analogue of deoxyuridine that should be a viable mechanistic inhibitor for both of these processes and, to that end, selected the 2′,4′-dideoxy-4′-methyleneuridine, dU*, (**1**) as our target for incorporation into single- and double-stranded DNA for biological evaluation [29]. Carbocyclic nucleosides have been used, *inter alia*, in enzyme studies on adenosine deaminase [30] and adenosine kinase [31]. In the present case for UDG, the replacement of the furanose oxygen by methylene would clearly inhibit the dissociative S_N1, or S_N1-like, process while its effect on an associative S_N2 process is also likely to impede glycolysis. How such a substrate analogue interacts with WT and mutant enzymes should lead to a clear understanding of the cleavage process and potentially of the basis of its prodigious selectivity.

The requisite carba-dU monomer (**2**), having a 5′-dimethoxytrityl group and a 3′-phosphoramidite, was prepared by a combination of standard methods [29] (Scheme 3). Carba-dU-modified oligonucleotides were synthesised using conventional phosphoramidite chemistry on a standard synthesiser. Sequences used for the cleavage studies included: d(TGC-CTA-AU*G-AGT-GAG), d(TGC-CTA-AUG-AGT-GAG), and d(TGC-CTA-ATG-AGT-GAG).



Reagents and conditions: i, 1 M H_2SO_4; ii, DMTCl, pyridine, DCM, DMAP, Et_3N; iii, $[(Pr^i)_2N]_2POCH_2CH_2CN$, 1*H*-tetrazole/$CH_3CN$.

Scheme 3. The synthesis of a 5′-protected 3′-phosphoramidite of carbadU.

T_m measurements on three synthetic pentadecadeoxynucleotides showed that the change from a central dT:dA base-pair to either a dU:dA or a carba-dU:dA base-pair results in the same lowering of T_m by 2°C. Since a base-mismatch would lead to a lowering [32] in T_m of some 6–7°C, this establishes that the carba-dU modification does not cause any loss of helix stability and that all three duplexes are fully intact under the conditions used in the subsequent enzyme hydrolysis studies. This result is in line with calculations and NMR studies [33] and with X-ray crystallographic data [34] on N-S conformational equilibria for carbocyclic nucleosides.

To show that the deoxyuridine nucleotide analogue resists enzymic hydrolysis, the three oligodeoxynucleotides were 5′-^{32}P-end-labelled and incubated with UDG enzyme. After treatment with alkali for strand scission at abasic sites, the products were analysed on a high percentage denaturating polyacrylamide gel which showed concentration-dependent cleavage for the oligodeoxynucleotide 5′-d(TGC-CTA-AUG-AGT-GAG) containing an unmodified deoxyuridine residue. In contrast oligomers containing the carba-dU migrated unchanged even after incubation with a two-fold molar excess of enzyme. Similarly, a dT-containing oligomer also fully resisted enzymic degradation.

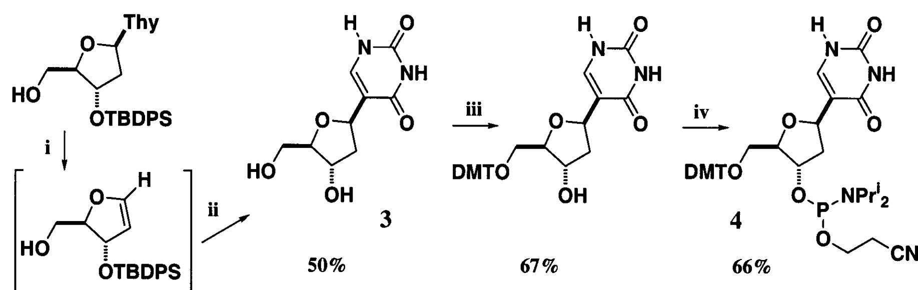
Gel-shift studies on the carba-dU oligomers with UDG suggested that these oligos have a somewhat reduced affinity for the enzyme. We therefore analysed the binding of both single-strand and double-strand oligos to UDG by surface plasmon resonance [35]. The affinity of WT enzyme for the oligomer containing the carba-dU residue, 5′-d(CCG-AAT-CAG-TTC-ACT-TCU*-AGC-CGA-GGT-ATT-TAG-CC), gave a K_d value 10^{-6} M, towards the upper limit of SPR analysis. By contrast, with carbadU DNA, the *inactive* mutants of UDG H210N and D88N display no changes in either the association or dissociation rates from those measured for dU-DNA, giving values of K_d 10^{-8} M. The results obtained for dsDNA duplexes were indistinguishable from those measured for ssDNA.

The resulting dU* oligomer is not itself cleaved by the UDG while both the regular deoxyribouridine and the 4′-thio-2′,4′-dideoxyribouridine residues are subject to glycohydrolase action [36] albeit with some retardation for 4′-thiodU. Thus, these results offer support for a mechanism of action of UDG that involves an S_N1 (or S_N1-like) process in which scission of the glycosylic bond involves transient formation of an oxocarbenium cation species at C-1′ of the deoxyribose residue, essentially stabilised by O-4′ (Scheme 2A). The slower reaction of the 4′-thio-dU is consistent with a reduced anomeric contribution from sulfur relative to oxygen in the furanose ring [28]. It is fully compatible with a role for the carboxylate residue Asp-88 (Asp145 in the human enzyme) as providing some measure of charge stabilisation of a transient oxocarbenium ion. In seeking to rationalise the observation that replacement of the 4′-oxygen by methylene leads to a 100-fold reduction in *affinity* of the WT enzyme for the carba-dU-containing oligomer, we considered the possibility that UDG has evolved to recognise the transition state corresponding to substrate cleavage and this enables the uracil residue to interact more effectively with amino acid residues in the affinity pocket identified in the X-ray structure of the product complex

[25]. An alternative explanation is that the slow conformational change that is involved in the discrimination between weak non-specific binding of UDG to dsDNA and tight binding to substrate [37,38] is retarded further in the case of the carbadU substrate to an extent that makes the BIAcore experiment unable to measure the true "on-rate" for binding.

It thus appears that UDG is potentiated for immediate scission of the glycosylic bond as soon as the active site pocket is occupied, which is itself a slow and highly selective process. When a uracil base appears in the UDG active site and fits into the precise hydrogen bonding network, it is cleaved immediately. Any structural modification to the deoxyuridine that disfavours the conformation required for such binding will therefore reduce the apparent affinity of the residue for UDG, and may do so differentially with respect to the mutant enzymes.

We therefore returned to the design of an alternative stable substrate analogue and selected deoxypseudouridine, dΨ, (**3**) for this purpose. Its C-nucleoside character endows it with strong resistance to glycosylic bond cleavage while it still retains the key furanosyl-O4′. The synthesis of the 5′-dimethoxytrityl-3′-phosphoramidite (**4**) was achieved by a combination of standard syntheses (Scheme 4). It was built into a duplex DNA decamer, 5′-d(CTGTΨATCTT) using an automated synthesiser with standard phosphoramidites, hplc purified, and the content verified by MALDI-TOF MS.



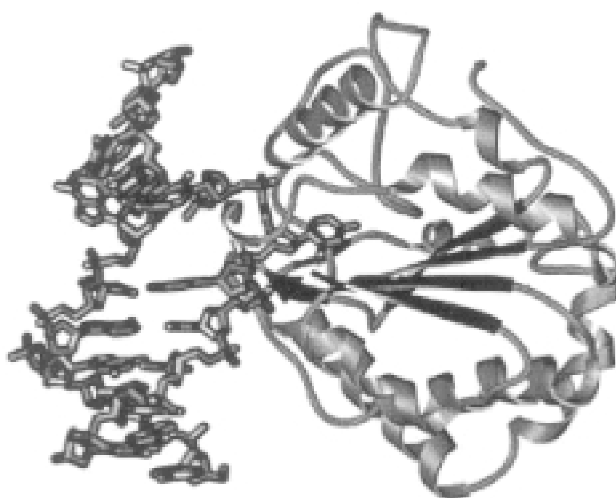
Reagents and conditions: i, HMDS, $(NH_4)_2SO_4$; $NaHCO_3$aq; ii, Uracil, $Pd(OAc)_2$, Ph_3As, DMF; TBAF, AcOH; $NaBH(OAc)_3$, AcOH; iii, DMTCl, py; iv, $(Pr^i_2N)_2$POCNE, Tet, DCM.

Scheme 4. The synthesis of a deoxypseudouridine 5′-protected 3′-phosphoramidite.

This decamer was fully resistant to base-excision by UDG in both single- and double-stranded forms. By contrast, a decamer of the same sequence but having a 4′-thiodeoxy-uridine at residue-5 was cleaved at about 1% of the rate of wild-type decamer. Most importantly, both of these decamers co-crystallised with human WT UDG to give a substrate complex for the duplex containing pseudodeoxyuridine (structure solved at 1.8 Å resolution) and a product complex for the 4′-thiodU (structure solved at 2.0 Å resolution) [39].

Compared to the structure of WT protein alone, these structures show that UDG undergoes a global conformational change from an 'open' unbound state to a 'closed' DNA-bound state in the UDG product complex, which evidently creates the catalyti-

cally-competent active centre [25]. In the product UDG-DNA complex, UDG has flipped its target uridine nucleotide out of the DNA base stack and into this active center, where the glycosylic bond is cleaved. This leaves an unstable and cytotoxic abasic site in DNA, which must be further processed [1] by at least AP endonuclease (APE1), polymerase β, and DNA ligase III (Scheme 5).



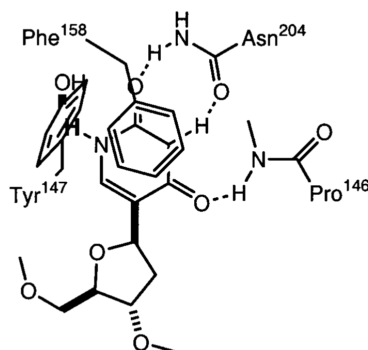
Scheme 5. B-DNA Duplex bound to UDG showing the "flipped-out" dΨ residue in the active site of the protein above a β-sheet and close to an Ω-loop containing several catalytic residues.

By contrast, UDG does not cleave the C–C glycosylic bond in dΨ (**3**) but provides an uncleaved, transition-state like UDG-DNA complex from which we can deduce an accurate chemical and structural mechanism for glycosylic bond cleavage by UDG. This is possible because the interchange of N1 and C5 between deoxyuridine and dΨ is isosteric, not affecting deoxyribose pucker, aromaticity, or hydrogen bonding functionalities of the substrate to the enzyme. The structure of the uncleaved "substrate" complex closely resembles the enzyme in the product complex, with a root-mean-square deviation of 0.26 Å for all Cα atoms. Thus UDG undergoes an architecturally determined conformational closure on binding its substrate while comparison of the substrate analogue and product complexes shows that the protein conformation is essentially unchanged throughout the reaction after it has "closed" on binding target DNA. Importantly, the UDG closing and coupled nucleotide flipping evidently funnels interaction energy into significant destabilisations of the substrate deoxyuridine *via* energetically demanding conformational distortions required by UDG active centre binding [39].

Double stranded DNA binds to UDG in the B-helical form, making close contacts with 4 phosphates in the strand containing the dU residue (Scheme 5). The deoxyuridine analogue is "flipped out" [17] of the DNA helix and into the enzyme active centre, which is set in a cleft between the β1 and β3 strands and partly formed by an Ω

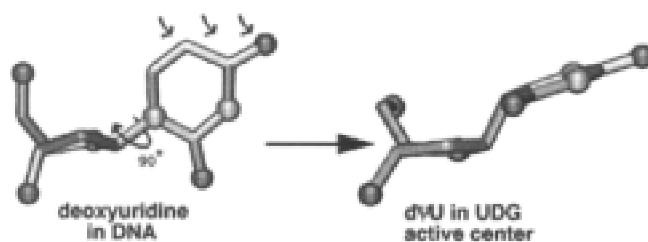
loop [25,40]. Three sets of interactions define a strikingly specific pocket for binding uracil that focuses the binding energy of the complex onto the deoxyuridine. First, direct hydrogen bond partners are available to every polar atom of the uracil ring. Second, a favourable face-to-face π-π stacking interaction is made with Phe158. Finally, Tyr147 prevents productive binding of thymine by edge-to-face proximity to the uracil ring.

Specific structural features discernible in the high-resolution 1.8 Å UDG uncleaved-substrate complex suggest a reaction mechanism in which the enzyme employs both steric and stereoelectronic effects to achieve catalysis. The extensive enzyme-DNA macromolecular interface allows UDG to constrain and orient the uracil ring to stretch and weaken the N–C$^{1'}$ glycosylic bond and simultaneously align orbitals for overlap from O4′ through the uracil O2 and/or O4. Moreover, these interactions are further enhanced after the glycosylic bond is cleaved when the cleaved uracil and DNA product complex achieves a higher degree of complementarity with the enzyme pocket than does the uncleaved dΨ DNA substrate complex (Scheme 6).



Scheme 6. Representation of dΨ bound in the active site of UDG showing hydrogen bonding to O2, NH3, and O4, edge-to-face interaction of Tyr147 with H5, and face-to-face interaction of Phe158 with the uracil ring.

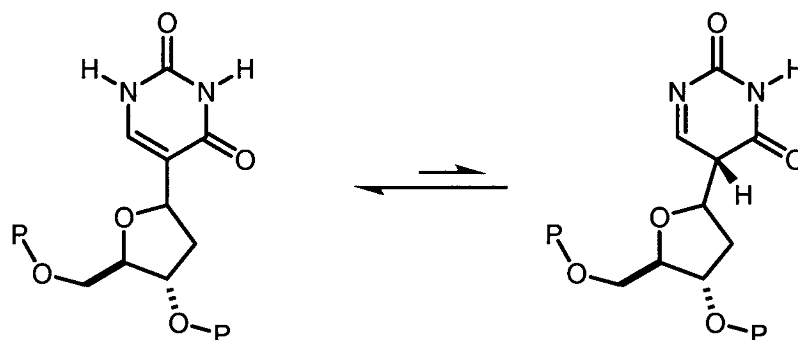
Prior to glycosylic bond cleavage, the normally trigonal planar C1 position (N1 in uracil) is distorted toward a tetrahedral geometry by the UDG active centre (Scheme 7). This well-defined tetrahedral distortion results from steric constraints imposed by the



Scheme 7. Change in conformation of dU on binding to UDG showing (i) flattering of the sugar pucker, (ii) rotation of the glycosylic bond by 90°, and (iii) pyramidalisation of Cl in the uracil ring.

extraordinary specificity and rigid walls of the well-defined UDG active centre, formed by Phe158 and Tyr147, coupled to the tight anchoring of the DNA phosphates both 5′- and 3′- of the uridine nucleotide by the UDG-DNA interface interactions (Scheme 5). These active-centre and phosphate binding residues are unlikely to yield as they are buttressed by the bulk of the protein. Thus, the distortion seen in the enzyme-DNA complex dΨ structure is almost certainly effected in the natural DNA deoxyuridine substrate.

This distortion of the dΨ in the active site clearly represents a high energy form for pseudodeoxyuridine, either as a result of pyramidal distortion or arising from tautomerisation of the uracil ring, thereby making C1 sp^3 hybridised (Scheme 8). Preliminary calculations show that both of these processes require around 10 kcal mol^{-1} of energy, which in the case of the imino tautomer arises largely as a result of loss of aromaticity. However, this distortion energy lies within the range of energy generated through DNA binding to UDG (*ca.* 9 kcal/mol). It is essential to appreciate that the same strain energy required to generate this high-energy dΨ tautomer would also be applied to the normal deoxyuridine substrate, primarily to distort its trigonal N1 towards tetrahedral geometry. As such the presence of the high-energy dΨ tautomer does not affect the mechanistic arguments developed here for the biologically-relevant deoxyuridine substrate but rather means that dΨ acts as a transition state analogue in its mode of binding into the active site of UDG.



Scheme 8. Tautomerisation of deoxypseudouridine to pyramidalise C1.

At this stage, it is not clear what *Gain* the enzyme achieves by imposing such *Strain* on its substrate. One possibility is that there is improved orbital alignment which effectively couples the release of electrons from O4′ into σ* antibonding orbital of the glycosylic bond (a dynamic anomeric effect). The uncleaved dΨ complex shows flattening of the sugar pucker to a mild C3′*exo*, thereby raising the glycosylic bond to a semi-axial position (Scheme 7). This would weaken the glycosylic C–N bond while simultaneously the electrons of the glycosylic σ-bond are delocalised into the π-system of the aromatic uracil ring [39]. That seems viable since the departing uracil enolate anion has a pK_a of 9.4, is entirely consistent with the absence of strong

general base catalysis in the neutral hydrogen bonding recognition features used by UDG to recognise the uracil base (Scheme 6). An alternative explanation may be that such distortion makes the reverse reaction, resulting from the collapse of the ion pair, more difficult thereby enabling capture of the sugar oxocarbenium ion by the tightly held water molecule that sits at 3.54 Å below the furanose ring. Further experiments will be needed to resolve this issue.

What is clear is that UDG funnels its binding energy for the macromolecular DNA substrate into catalytic power by conformationally closure to enforce substrate distortions. Such a process would be energetically difficult in small molecule systems that lack large interfaces and cannot control transitions between two distinct, rigid conformational states. The architecturally stabilised closed UDG conformation and active centre structure may well couple two distinct yet complementary stereoelectronic effects that promote efficient catalysis by altering three orthogonal, non-overlapping electron orbitals into stereochemically defined conformations allowing the required electron transpositions for glycosylic bond cleavage (Fig. 1).

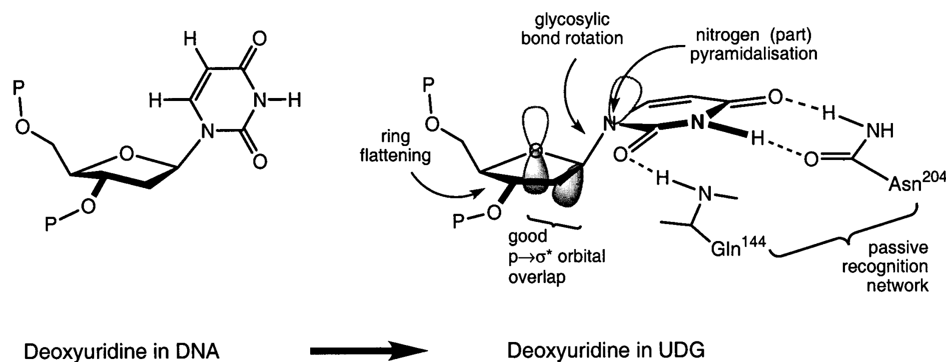


Figure 1. Strain features applied to a deoxyuridine residue in a B_helical conformation to achieve location in the active site pocket of UDG. Glycosylic bond cleavage is accelerated by some 10^{12} in consequence.

There is no doubt that the bond-breaking process is dissociative in character [39]. Our experimental 1.8 Å co-crystal structure shows that the active centre Asp145 is in the same closed conformation as in the uncleaved substrate complex in the product complex. A catalytic-centre water is tightly positioned 3.54 Å below the deoxyribose C1′ by four hydrogen bonds with only modest general base catalysis, so it is likely a poor nucleophile. Following dissociation of the incipient deoxyribose oxocarbenium ion from the uracil ring, this water is well-positioned to become the 1′-α-OH by trapping the oxocarbenium ion. The UDG-DNA product structure also shows that the uracil tilts following cleavage to move deeper into the active site, shortens hydrogen bonds, and improves its stacking interaction with Phe158. Finally, in the cleaved product complex, the abasic nucleotide relaxes to a more puckered C2′-*endo* form and withdraws slightly from the enzyme thereby reducing sterically-induced strain.

It is apparent that the experimentally-defined product complex is significantly less strained than the experimentally-defined uncleaved-substrate complex.

These structural results are wholly in line with biochemical results showing that the enzyme binds preferentially to its cleaved product [40]. Such experimental DNA binding measurements indicate that human UDG binds with 11.2 kcal/mol of binding energy to product, but with only 8.8 kcal/mol binding energy to its uncleaved substrate. Calculations in progress [41] suggest that this is about the degree of strain energy required to achieve a measure of pyramidalisation prior to a favoured unimolecular dissociation of the glycosylic bond. All of these results are fully consistent with a UDG nucleophilic substitution reaction that is dissociative in nature, involving substrate strain induced by the macromolecular interface coupled to the formation of a stable uracil enolate anion and a transient oxocarbenium ion that is subsequently captured by a specific water.

These conclusion are challenging. How do they relate to other results? Based on kinetic isotope effects and computational analysis, Vern Schramm has recently demonstrated that depurination of RNA by the Ricin A chain involves a dissociative glycosylic bond cleavage of S_N1 character [42]. James Stivers and Ben Horenstein have used primary and secondary kinetic isotope effects with UDG to reach a like conclusion, namely that the reaction is dissociative in nature and generates an oxocarbenium ion species.

What further problems remain to be resolved? The interpretation of the pyramidalisation of dΨ requires a knowledge of the tautomeric state of the uracil in that residue. The question of whether more charge is localised onto O2 or O4 of the uracil ring may best be resolved by computational methods. The precise point for the transition state in bond-breaking from N1 to C1′ may also be best resolved by computation. The 10^7 discrimination against deoxycytidine residues is not properly explained. Perhaps the dominant question is "How is the proton translocated from the captive water molecule the deoxyribose to the uracil enolate anion above the ribose plane?" There is no obvious amino acid residue to do this if, as claimed [44], His-187 is uncharged in the active site. Perhaps only with these questions answered will we be able to understand an enzyme that uses *Strain* for *Gain*!

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