Contrasting effects of mutating active site residues, Aspartic acid 64 and Histidine 187 of *Escherichia coli* uracil-DNA glycosylase on uracil excision and interaction with an inhibitor protein

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Uracil, a promutagenic base, arises in DNA by spontaneous deamination of cytosine or by the malfunctioning of DNA polymerases. To maintain the genomic integrity, cells possess a highly conserved base excision repair enzyme, uracil-DNA glycosylase (UDG). UDGs have a notably high turnover number and strict specificity for uracil in DNA. UDGs are inhibited by a small proteinaceous inhibitor, Ugi, which acts as a transition state substrate mimic. Crystal structure studies have identified the residues crucial in catalysis, and in their interaction with Ugi. Here, we report on the mutational analyses of D64 (D64H and D64N) and H187 (H187C, H187L and H187R) in the active site pocket of *Escherichia coli* UDG. The mutants were compromised in uracil excision by ~200-25,000 fold when compared to the native protein. In contrast, our analysis of the in vitro formed UDG-Ugi complexes on urea gels shows that D64 and H187 contribute minimally to the interaction of the two proteins. Thus, our findings provide further evidence to the primary function of D64 and H187 in catalysis.

Uracil, a natural component of RNA, arises in DNA either due to deamination of cytosine or by occasional incorporation of dUMP by DNA polymerases. Occurrence of uracil in DNA is promutagenic and if left unrepaired, leads to GC → AT mutations. However, to safeguard the informational content of the genome, cells have evolved with uracil-DNA glycosylase (UDG)\(^1,2\). UDGs have been identified from a variety of sources of prokaryotic and eukaryotic origin and these enzymes show a high degree of conservation\(^3\). The UDGs also interact with a number of proteins such as the *Bacillus subtilis* phage encoded uracil DNA glycosylase inhibitor, Ugi, and the cellular factors such as single stranded DNA binding protein\(^4,5\). Ugi (9.4 kDa), is an early gene product of the *Bacillus subtilis* bacteriophage (PBS-1/-2), which forms a highly specific and extremely stable complex with the conserved class of UDGs in 1:1 stoichiometry\(^6\). The complex of *Escherichia coli* UDG (EcoUDG) and Ugi is irreversible under physiological conditions. However, it can be dissociated by treatment with SDS, or 8 M urea at 70 °C. Thus, UDGs constitute a remarkably interesting model system to understand the basis of catalytic prowess and specificity associated with protein-DNA and protein-protein interactions.

The crystal structures of several UDGs or their complexes with DNA or the Ugi, have suggested a prime role for the highly conserved D64 and H187 in catalyzing (numbering according to *E. coli* UDG) the glycosidic bond cleavage (see Discussion)\(^6-11\). Interestingly, these residues are differently oriented in *Eco*UDG and warrant independent mutational analyses\(^10,11\). The UDG-Ugi crystal structures have shown that the highly conserved sequence motifs in UDGs, such as the water activating loop, 62-GQDPYH-67; the Pro-Ser loop, 84-AIPPS-88; and the DNA intercalation loop, 187-HPSPLS-192 which lie at the interface with Ugi contribute to the stability of the complex through the interactions at the hydrophobic cavity (between the α2 helix and the antiparallel β-sheet) or the β1-edge residues (Q19, E20, S21, L23 and E28) of Ugi. The key UDG residues such as Q63, D64, Y66, H67, and H187 in the active site pocket of UDG make direct or water mediated H-bonds with the Ugi β1-edge\(^10,11\).

Mutational analysis of D64 (D64N) and H187 (D, G) in *Eco*UDG as well as human and viral counterparts has earlier shown that these mutants are highly compromised in uracil excision\(^12,13\). To further assess the significance of these residues in catalysis, in this study, we have included a different set of mutations at
D64 (H, N) and H187 (C, L, R). In addition, we exploited these mutants to gain insights into their interaction with Ugi, a transition state substrate mimic.

**Materials and Methods**

**Oligodeoxyribonucleotides and radioisotopes**

Oligodeoxyribonucleotides were obtained from Ransom Hill Bioscience, USA. [γ³²P] ATP was obtained from DuPont NEN, USA.

**Generation of D64 mutants**

The UDG gene fragment encompassing D64 was released from pTZUng4S¹¹ as an EcoRI-HindIII fragment and subcloned into pTZ18R between the same sites (pTZUng4S'). The single stranded DNA from pTZUng4S' and the oligomer, 5′-ctgcggcag(a/e)atccctatcaac-3′, were used to generate D64N and D64H mutants by a modified Kunkel's mutagenesis protocol¹⁵,¹⁶ and confirmed by DNA sequencing.¹⁷ The mutation containing regions from pTZUng4S' constructs (50 ng) were PCR amplified by a gene specific (5′-cgcggattaactctatcaac-3′) and a vector specific (5′-gggctctaggggaac-3′) primers (20 pmol each) using Pfu DNA polymerase. The PCR was carried out for 25 cycles of incubations at 95°C for 45 s, 50°C for 30 s and 72°C for 1 min after initial denaturation at 95°C for 1 min. The final extension was allowed at 72°C for 10 min. The resulting PCR products were digested with Ncol and Nrl and used to replace the Ncol-Nrl (~450 bp) fragment from pTrcEcoUDG (Fig. 1). The mutations in pTrcEcoUDG were confirmed by DNA sequencing.

**Generation of the H187 EcoUDG mutants**

The single stranded DNA template derived from pTZUng2B¹⁶ and the mutagenic oligomers 5′-cgcggagaagcttctt-3′; 5′-cgccgagggatcttctt-3′; and 5′-cgcggagaagcttctt-3′ were used to obtain H187L, H187R and H187C mutants, respectively and confirmed by complete nucleotide sequencing. The Nrl-HindIII fragments (~550 bp) were excised from the pTZ constructs and subcloned into similarly digested pTrcEcoUDG to generate expression constructs.

**Purification and estimation of the D64 and H187 mutants**

The wild type and the various UDG mutants (D64N, D64H, H187L, H187C and H187R) were purified to apparent homogeneity by chromatography on Superdex G-200, MonoS and heparin Sepharose columns.¹⁶,¹⁸ The purity of proteins was checked by electrophoresis on SDS (0.1%) polyacrylamide (15%) gel and estimated by Bradford’s reagent using BSA as standard¹⁷.

**Assay of uracil excision activity**

The DNA oligomer, SSU4 (5′-acucatagtctctgtaaat-3′) was 5′ [³²P]-end labelled and purified on Sephadex G 50 minicolumns.²⁰ Reaction mixtures (70 µl) containing 35 pmol of the oligomer in UDG

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Fig. 1—Diagrammatic sketches of various constructs used for in vitro production of UDG. (A): Expression constructs for wild type and the UDG mutants. (B): Bicistronic constructs used for simultaneous expression of UDG mutants (D64) and Ugi. (C): Constructs used for simultaneous expression of UDG mutants (H187) and Ugi.
buffer (50 mM Tris-HCl, pH 7.4, 1 mM Na₂EDTA, 1 mM DTT and 25 μg/ml BSA) were supplemented with 5 μl of appropriate dilution of UDG (25 pg of wild type enzyme, 25 ng of H187L, 250 ng of H187R, 25 ng of H187C, 250 ng of D64H and 2.5 ng of D64N) and incubated at 37°C for 10 min. The percent uracil excision activity of the mutants was calculated with respect to wild type UDG\(^\text{16}\).

**Bicistronic constructs for overproduction of UDG-Ugi complexes**

The bicistronic constructs of Ugi with the D64 mutant UDGs in pTrc99C expression vector, were generated as described\(^\text{16,18}\). The bicistronic constructs of the UDG mutants (H187) were also the same except that pET11d expression vector was used (Fig. 1). Overproduction of the complexes was carried out\(^\text{16,18}\) in E. coli TG1 and E. coli BL21(DE3) for the D64 and H187 UDG mutants, respectively.

**Analysis of in vivo formed UDG-Ugi complexes**

The cell free extracts of the recombinants harboring bicistronic constructs of Ugi with D64H, D64N, H187L, H187R and H187C mutants, were made after induction of mid log phase cultures with 0.5 mM IPTG and analysed on 15% polyacrylamide (19:1 crosslinking) gels without or with different concentrations of urea\(^\text{16}\) and stained with Coomassie brilliant blue\(^\text{21}\).

**Results**

**Generation of expression constructs for UDG, and UDG-Ugi**

Fig. 1 shows diagrammatic sketches of the various expression constructs used in overproduction of the wild type and the D64H, D64N, H187C, H187L and H187R mutants of EcoUDG. The bicistronic constructs used in co-expression of various UDGs and Ugi, for the formation of the UDG-Ugi complexes in cellular milieu are also shown.

**Overexpression and purification of UDG**

The pTrc99C based expression constructs (Fig. 1) were transformed into an ung-strain of E. coli BW310, and the UDG purified as described in Materials and Methods. Analysis of the pooled fractions from the heparin Sepharose column, on SDS-PAGE (Fig. 2) showed that the proteins were purified to apparent homogeneity.

**Uracil excision by H187 and D64 mutants**

Uracil excision activities of the various mutants were determined using an oligodeoxyribonucleotide, SSU4 containing uracil at the fourth position from the 5'-end, as substrate. As shown in Table 1, relative to the wild type protein, all the mutants at the D64 and H187 positions were compromised ~200 to 25,000 fold in their uracil excision activity. Among the mutants, the D64N with an activity of ~0.5% was the most active whereas the H187R with an activity of ~0.004% was the least active.

**Analysis of the stability of the in vivo formed UDG-Ugi complexes**

We have earlier described an assay\(^\text{16}\), wherein the cell free extracts of the transformants containing in

![Fig. 2](image)

**Table 1**—The relative activity of uracil excision by the wild type, D64 and H187 UDGs

<table>
<thead>
<tr>
<th>UDG</th>
<th>pmoles of uracil released per μg of UDG per min (×10⁶)</th>
<th>Relative activity of UDG per min (×10⁶) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>530</td>
<td>100</td>
</tr>
<tr>
<td>D64H</td>
<td>0.4</td>
<td>0.0075</td>
</tr>
<tr>
<td>D64N</td>
<td>25.7</td>
<td>0.4850</td>
</tr>
<tr>
<td>H187C</td>
<td>8.4</td>
<td>0.1584</td>
</tr>
<tr>
<td>H187L</td>
<td>4</td>
<td>0.0755</td>
</tr>
<tr>
<td>H187R</td>
<td>0.2</td>
<td>0.0037</td>
</tr>
</tbody>
</table>
vivo formed complexes of the two proteins are electrophoresed on polyacrylamide gels containing different concentrations of urea (0 to 8 M) to determine the stability of the UDG-Ugi complexes. In such gels, the UDG-Ugi complex (35 kDa) with a pI of 4.9 migrates in the middle of the gel as a distinct band.

Analysis of the cell free extracts containing in vivo formed complexes of Ugi with the UDG mutants is shown in Fig. 3. Expectedly, the complexes of Ugi with the D64H and D64N (Fig. 3 A) and H187L, H187C, and H187R (Fig. 3 B) mutants co-migrate with the complex of wild type proteins (compare lanes 2 and 3 with 1, Fig. 3 A; or lanes 3 to 5 with 2, Fig. 3 B). Interestingly, the complexes of Ugi with the mutant UDGs were observed to be completely stable in 6 M urea (compare panels i and ii, Figs. 3 A and 3 B). The complexes of Ugi with the wild type, D64H (lanes 1 and 2, Fig. 3 A, panel iii) or the H187C and H187R (lanes 4 and 5, Fig. 3 B, panel iii) UDGs were stable to even 8 M urea. However, as seen from the diminishing intensity of the UDG-Ugi band, some dissociation of the complexes of D64N and H187L UDGs occurred at 8 M urea. Dissociation of these complexes in 8 M urea was also supported by the presence of a smear below the UDG-Ugi complexes. The smear corresponds to Ugi that results from dissociation of the complex. Because of its acidic pI (4.2) and low molecular weight (9.4 kDa), Ugi migrates faster than the complex (lane 6, Fig. 3 B). While the pure Ugi migrates as a sharp band, its presence in the form of a smear as well as its retarded mobility is indicative of the fact that the complexes dissociated while they electrophoresed through the gel. Further, as described earlier, the presence of a sharp band corresponding to free Ugi in lanes 2 and 3 of the native as well as 6 M and 8 M urea gels (Fig. 3 A) is due to the expression of Ugi in molar excess to the UDG from the pTrec99C based bicistronic expression constructs. It may also be mentioned that a band or smear representing UDG which would have arisen from the dissociation of the complexes in these lanes is not expected to be detectable. Because of its near neutral pI (6.6) it migrates near the well (lane 1, Fig. 3 B) where a large number of cellular proteins also fractionate. The analysis of the purified complex of the wild type proteins (Fig. 3 A, lane 1) provided a

Fig. 3—Analysis of the in vivo formed complexes of Ugi with UDG [The cell free extracts (~10 μg of total proteins) from the transformants harboring the bicistronic constructs for Ugi and the various UDG mutants were analyzed on 15% polyacrylamide gels containing no urea (panel i); 6 M (panel ii) or 8 M (panel iii) urea.] (A): Analysis of the complexes of Ugi with D64 mutants. Lanes: 1, EcoUDG-Ugi complex marker; 2 and 3, cell free extracts harboring bicistronic constructs of Ugi with D64H and D64N UDGs, respectively; 4, Ugi marker. (B): Analysis of the complexes of Ugi with H187 mutants. Lanes: 1, UDG marker, lanes 2 to 5, cell free extracts harboring bicistronic constructs of Ugi with wild type, H187L, H187C and H187R UDGs, respectively; 6, Ugi marker.
marker for the UDG-Ugi complex. On the other hand, the analysis of total cell extract containing the complex of the wild type proteins (Fig. 3, lane 2) served as a control to rule out dissociation of the Ugi complex with H187L in 8 M urea due to some unknown factors in the cell extracts. It may also be noted that with the increasing urea concentration in the gels, the relative mobility of the UDG-Ugi and Ugi bands changes. This is because in the native gels the Ugi is in its native conformation, which migrates faster on the gel, whereas in the presence of urea the Ugi unfolds and migrates slower. On the other hand, the UDG-Ugi complex is recalcitrant to urea, and retains its tertiary structure.

Discussion

Crystal structures of several UDGs and their complexes with the substrate DNA or the substrate mimic, Ugi have provided major clues to the mechanisms that regulate extreme substrate specificity and catalysis by the conserved class of UDGs. Accordinng to the proposed mechanisms a water molecule is activated by D64 to launch an attack on to C1’ of the sugar leading to formation of oxocarbenium cation and uracil anion (associative mode of bond cleavage). Subsequently, the departure of uracil is facilitated by a hydrogen bond donated to O2 by His 187. More recently, a dissociative mode of catalysis has been proposed wherein the glycosidic bond cleavage occurs by an autocatalytic step facilitated by the interactions between the Ser-Pro loops of the enzyme with the backbone phosphates in the substrate, and the attack by the water molecule occurs subsequent to the bond cleavage. However, in both the proposed mechanisms, the D64 and H187 play a crucial role in catalyzing the cleavage of the N-glycosidic bond. Consistent with proposed mechanism and earlier mutational analysis, our results suggested that D64 and H187 are essential for efficient catalysis.

Recently, the mismatch specific uracil DNA glycosylase (MUG) which bears striking architectural homology to the UDGs has been characterized. Although the physiological substrate for this enzyme might be ethenocytosines in DNA, it does excise uracil from G:U mismatches at an extremely sluggish rate. Interestingly, the equivalent of the catalytic D64 of the UDGs in the water activating motif (GQDPY), in MUG is represented by an Asn in the topologically equivalent motif, GINPG. It has been suggested that Asn can also position the water molecule and activate it but extremely poorly as compared to the conserved class of UDGs. Although, the D64N mutant is compromised about 200 fold in its activity, the observation that it is far more active than the D64H mutant supports the proposed role of Asn in MUG class of uracil DNA glycosylases.

On the basis of the studies carried out for the HSV-1 and 2 UDGs, it was believed that the protonation of the O-2 of the uracil is carried out by the conserved histidine present in HPSPLS, acting as a general acid. Mutational studies with EcoUDG, however, have shown that this conserved histidine does not act as general acid, but is instead neutral and acts as an electrophile. On this basis, it was proposed that the electrophilic interaction stabilizes the developing enolate on the uracil O-2 in the course of its excision. We expected that such a role could be fulfilled by Cys in the H187C mutant. However, the observation that the H187C mutant was more than 600 fold compromised in uracil excision, suggests that the interaction of H187 with the O2 or uracil of the uracilate anion is highly directional. And, therefore it is not surprising that the H187R and H187L mutants, wherein the side chains of Arg and Leu will fail altogether to substitute for such a function, were more than a 1000 fold worse in their uracil excision activity. Interestingly, the topological equivalent of His of HPSPLS motif of UDG in MUG is an Asn in the PNPGL motif. Although, none of our mutations contained Asn at H187, the observation that all three mutants tested were extremely compromised in uracil excision, supports that lack of the equivalent of H187 in MUG also contributes to its extremely inefficient activity in uracil excision.

In this study, we have also assessed the effect of mutations in the active site residues of UDG on their interaction with Ugi. Crystal structure showed that H187 of the DNA intercalation loop, makes hydrophilic contact with I22 and M56 of Ugi. Also, the H187 main chain N establishes a water-mediated hydrogen bond with the E28 O of Ugi. However, this interaction does not appear to be highly conserved as the HSVUDG-Ugi co-crystal does not reveal this interaction. Our observation that the substitutions at H187 with Arg, Cys or Leu do not substantially alter the stability of the UDG-Ugi complexes suggests, a minimal role for the His at this position. Further, the crystal structure suggests that the D64 interacts via the side chain O2-mediated hydrogen bond.
with main chain S21 O of Ugi. Thus, our observation that the complexes of Ugi with D64H as well as D64N are also highly stable suggests that even this interaction is not critical for the stability of the complex. While the possibility of compensatory interactions with the side chains of Asn or His at this position has not been ruled out, in the light of the observation that S21 side chain O' is fixed by an additional hydrogen bond with the side chain of H67 of UDG, such a possibility appears unlikely.

In conclusion, our observation of the mutants at the D64 and H187 positions of UDG are in agreement with the predictions from the crystal structure studies and provide additional evidence for the sluggish nature of the MUG enzyme. More importantly, in this study, for the first time, we exploited the UDG mutants at D64 and H187 to gain insights into their interaction with Ugi. The mutations severely hamper the catalysis of uracil excision from DNA. On the contrary, even though Ugi is a substrate mimic of UDG, the mutations have minimal effect on the stability of their complexes with Ugi.

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