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**REVIEW**

# Pathways of Accumulation and Repair of Deoxyuridine Residues in DNA of Higher and Lower Organisms

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**Abstract**—Uracil DNA glycosylase hydrolyzes the N-glycosidic bond between sugar phosphate backbone and uracil residue appearing as the result of spontaneous deamination of cytosine or during wrong incorporation of dU residues during DNA synthesis. Uracil DNA glycosylases are very conservative enzymes. They have been recognized in all pro- and eukaryotic organisms and also in pox and herpes viruses. This review highlights the pathways of accumulation of uracil and its derivatives in DNA, the main physicochemical and biochemical properties of uracil DNA glycosylase, and regulation of its functioning. Special attention is paid to detailed mechanisms of recognition and removing of damaged (or wrong) base by uracil DNA glycosylase. These mechanisms have been validated by the methods of X-ray analysis and kinetic and thermodynamic approaches.

*Key words*: accumulation of deoxyuridine in DNA, pathways of repair, uracil DNA glycosylase, structure, mechanism of action

## EXCISION REPAIR AND PATHWAYS OF ACCUMULATION AND REMOVAL OF DEOXYURIDINE RESIDUES

The structural integrity of DNA is constantly subjected to the effects of various endogenous and exogenous agents [1, 2]. Cells possess several protective mechanisms acting at various levels; these mechanisms prevent and/or repair DNA damages [1-9]. DNA repair is closely related to numerous biological processes including gene transcription and regulation of the cell cycle. Several repair mechanisms are recognized. They include direct repair, base and nucleotide excision repair, mismatch repair, and post recombinant repair. Some of these mechanisms have been analyzed in several monographs and reviews [1-10].

Excision repair represent a common mode for removal of DNA lesions. This process requiring concerted action of several enzymes consists of several steps: 1) recognition of a defect (damage); 2) incision of DNA chain near this defect (damage); 3) removal of the defect (damage) and local DNA degradation; 4) replication of removed DNA site; 5) ligation of repaired DNA chain [1-9]. In the case of excision repair the N-glycosidic bond between sugar residue and damaged base is cleaved and this provides high specificity of removal of the damaged base; however, this requires the presence of numerous glycosylases sensitive to a wide range of damages [1-7]. Some of these glycosylases have been considered in several reviews [1-10].

Uracil DNA glycosylase (UDG; EC 3.2.2.3) represents a class of highly conservative and well studied enzymes of excision repair. They have been found in all organisms. This enzyme catalyzes uracil removal from DNA. Some aspects of UDG functioning have been previously analyzed in several monographs and reviews [1-9] considering repair enzymes. Data on structure and functions of UDG have been reviewed in [6, 8]. In the present review we consider pathways of accumulation of deoxyuridine in DNA of higher and lower organisms and mechanisms underlying its removal catalyzed by UDG. We also analyze recent studies on UDG properties which were investigated by using the method of X-ray analysis and by kinetic and thermodynamic approaches.

**Spontaneous and chemical deamination of cytosine.** Spontaneous deamination of cytosine is one of the ways for the appearance of uracil in a DNA molecule. In solution two pathways of cytosine deamination exist at neutral pH values [11, 12] (Fig. 1). The first includes direct attack of the fourth position of the pyrimidine by hydroxyl-ion. Perhaps this is the main mode of cytosine deamination under physiological conditions. The second pathway

*Abbreviations*: UDG) uracil DNA glycosylases.

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occurs via dihydrocytosine formation as an intermediate. Such type of deamination may be referred to as the chemically induced mechanism. Increase or decrease of pH increases cytosine deamination [12]. Studies of the temperature dependence of the cytosine deamination revealed that in single stranded DNA the rate constant of this reaction is $2 \cdot 10^{-10}$ sec$^{-1}$ at 37°C [13]. In double stranded DNA the rate of cytosine deamination *in vitro* is only 0.3-0.5% of the rate of its deamination in single stranded DNA.

Although amount of single stranded DNA of a living cell remains unclear, it is evident that the appearance of single stranded DNA sites during replication, recombination, and transcription is accompanied by local DNA denaturation and this increases the possibility of cytosine deamination. Recently, it has been shown that transcription causes 4-fold increase in the frequency of cytosine deamination in the coding (non-transcribed) chain of DNA. Moreover, A–T rich DNA duplexes are subjected to spontaneous local denaturation, known as "respiration", which may increase deamination rate [14]. The frequency of GC→AT transitions during spontaneous mutations in ung$^-$ bacteria *in vivo* was $3.2 \cdot 10^{-13}$ sec$^{-1}$. Assuming that the human genome consists of $6 \cdot 10^9$ bp and cytosine represents about 20%, the daily rate of cytosine deamination should be about 60-120 cytosines per genome [15].

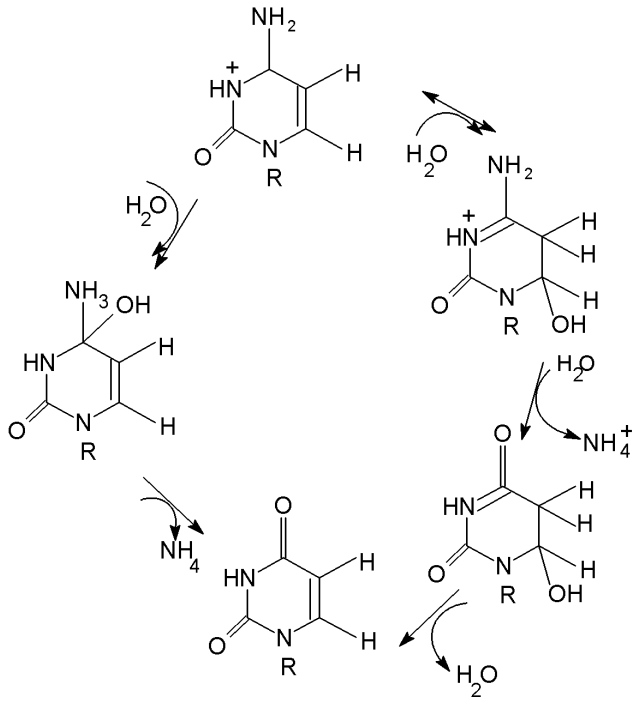

**Fig. 1.** Pathways of cytosine deamination.

If cytosine deamination involves formation of dihydrocytosine and dihydrouracil as intermediates, each of them may represent a separate form of DNA damage, which could be repaired by mechanisms that differ from that of uracil repair.

Cytosine deamination may be induced by various chemical agents such as nitrous acid [16] and sodium bisulfite [17]. The first is a rather nonspecific reagent because it also causes adenine and guanine deamination [18] and promotes formation of cross-links of DNA chains [19]. Nitrous acid attacks cytosine residues of double stranded DNA with nearly the same efficacy as in single stranded DNA. Sodium bisulfite deaminates only cytosine and only in single stranded regions of DNA. This reagent converts cytosine into uracil with formation of intermediates: 5,6-dihydrocytosine-6-sulfonate and 5,6-dihydrouracil-6-sulfonate; at alkaline pH values they are converted into uracil [17]. The other mechanism of conversion of cytosine into uracil in DNA molecules involves DNA treatment with strong alkali [20].

**Uracil formation from analogs of nitrogenous bases.** Some bases which are absent from DNA under normal conditions share similarity with normal (unmodified) nitrogenous bases. These analogs may be included into the growing DNA chain by DNA polymerases. For example, insertion of a thymine analog, 5-bromouracil, into DNA followed by subsequent light irradiation of DNA with wavelength 313 nm in the presence of cysteamine resulted in conversion of 5-bromouracil into uracil [21].

In mammalian cells most of cytosines of CpG sequences are methylated by cytosine-5-methyl transferase after replication [22]. Bacterial Hpa II methyl transferase and Dam methylase methylate internal cytosines of certain nucleotide sequences. Bacterial and mammalian methyl transferases require S-adenosyl methionine (SAM) as a methyl group donor. In the absence of SAM, Hpa II methyl transferase deaminates cytosine and this increases the number of mutations [23]. It is suggested that 5-methyl transferase binding at C-6 of cytosine forms the anionic intermediate "dihydrocytosine–enzyme". In the presence of SAM this complex is rapidly methylated with formation of 5-methylcytosine. Under conditions of SAM deficit cytosine undergoes deamination leading to C→U transitions. Although enzymatic deamination of cytosine contributes to uracil formation (and consequently to C→T transitions) this process is not the major cause of mutations in sites of methylated cytosines [24]. However, it was demonstrated that overexpression of cytosine-5-methyl transferase leads to significant increase in C→T transitions in non-canonic sites [25].

**Errors in DNA synthesis.** dUTP is a natural cellular metabolite. Under normal conditions it is metabolized by deoxyribouridine triphosphatase. However, in cells a relatively stable level of dUTP exists. Uracil may be inserted into DNA during replication. This may occur in organ-

isms normally containing thymine and in some viruses where uracil totally replaces thymine in DNA. In the former case the presence of U–A rather than T–A pair should not affect accuracy of DNA synthesis but it may impair specific binding of various enzymes and regulatory factors. Small amounts of dUMP are inserted during DNA synthesis in *E. coli* and other prokaryotes like *B. subtilis* [26-28]. Incorporation of dU residues strictly depends on the intracellular level of dUTP because DNA polymerase (e.g., *E. coli* DNA polymerase III) often has almost equal $K_m$ value for dUTP and dTTP [27]. In wild type *E. coli* cells UDG removes uracil from DNA. However, UDG lacking mutants (ung$^-$) are characterized by the frequency of dUMP insertion into DNA of one nucleotide per (2-3)·10$^3$ nucleotides [27]. In double mutants (ung$^-$, dut$^-$) deficient in UDG and deoxyribouridine triphosphatase (the latter enzyme prevents dUTP hydrolysis to dUMP and consequently increases intracellular level of dUTP) the frequency of U appearance in DNA may reach 0.5% of all bases [27]. Infection of mutants with T4 bacteriophage results in substitution of about 30% of phage thymines for uracil [28].

It is possible that most if not all organisms synthesizing TMP from dUMP insert some amount of uracil into DNA during replication. In bacteria dUMP is mainly formed from dUTP and therefore dUTP normally exists in cells. In mammalian cells dUMP is generally formed from dCMP and it is rapidly converted into triphosphate. In human lymphocytes inhibition of TMP synthesis from dUMP increases the dUTP level versus TTP. This increases probability of uracil incorporation instead of thymine. For example, in the reaction of TMP synthesis from dUMP catalyzed by thymidylate synthetase one carbon unit (methyl group) is transferred from $N^5$,$N^{10}$-methylenetetrahydrofolate to dUMP. Resultant dihydrofolate is reduced again by the enzyme dihydrofolate reductase. This regeneration of tetrahydrofolate is essential for continuation of TMP synthesis (as well as many other processes). Dihydrofolate reductase is inhibited by 4-amino-10-methylfolate (methotrexate) [29-31]. Treatment of cells with methotrexate caused an increase in dUMP level with simultaneous reduction of TTP content [32-34]. This resulted in further impairments of pyrimidine metabolism. Finally, intracellular level of dUMP in human lymphocyte cell culture increased by 1000-fold and in spite of absence of deoxyribouridine triphosphatase dUTP content increased by three orders of magnitude [31, 32]. The content of TTP in the cells decreased by 50 times and this (together with significant increase of dUTP level) promotes dUMP incorporation into DNA (Fig. 2).

Normally DNA of bacteriophages PBS 1 and PBS 2 contains uracil instead of thymine [34]. Infection of *B. subtilis* cells with these phages induces many enzymes including deoxyribothymidine monophosphatase, deoxyribocytidine triphosphate deaminase, and deoxyribouridine monophosphate kinase [35]. These enzymes alter the normal pathway of deoxyribonucleotide triphosphate biosynthesis in host cells and increase dUTP level versus TTP. This promotes insertion of uracil instead of thymine into DNA.

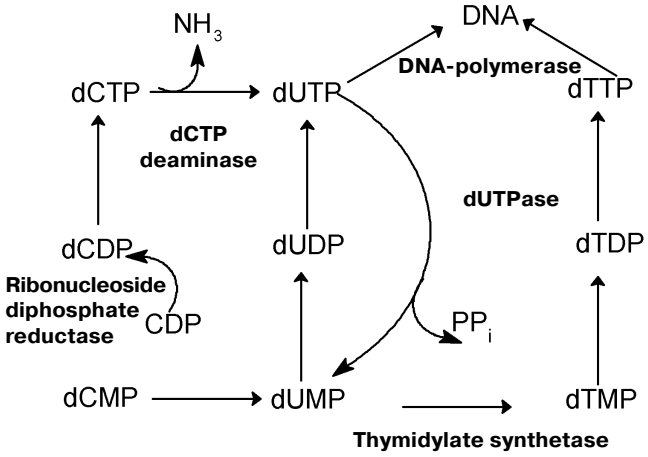


**Fig. 2.** Chemical pathways of dUTP and dTTP formation [31, 32].

**Some characteristics of uracil DNA glycosylase.** UDG was first discovered in *E. coli* cell extracts in 1973 [36]. Later this enzyme was found in many organisms including *B. subtilis* [37], wheat germ [38], *Dictyostelium dyscoideum* [39], *Saccharomyces cerevisiae* [40], pox and herpes viruses [41, 42], and hyperthermophilic microorganisms [43, 44]. UDG was also found in numerous eukaryotic (including human) cells and tissues such as rat liver [45], calf thymus [46, 47], human fibroblasts [48], *Hela* cells [49, 50], peripheral lymphocytes [51], KB-cells [52], stem cells of patients with acute myeloid leukemia [53], and human placenta [54]. All known UDGs are monomeric globular proteins with molecular masses ranged from 18 to 50 kD (depending on a source of this enzyme) [55, 56]. The usual interval of UDG molecular masses is within the range from 27 to 35 kD. The number of amino acid residues varies from 199 to 359. The N-terminus rather variable by both length and amino acid composition is possibly required for particular intracellular localization of the enzyme.

The optimum of pH varies from 7.0 to 9.0. Functioning of this enzyme does require cofactors, and it is active in the presence of the chelating agent EDTA [1-8, 55, 56].

Genes encoding UDG in *S. cerevisiae*, *E. coli*, herpes simplex type I and II, mouse, man, and other organisms have been cloned [57-60]. UDG is a conservative enzyme. For example, herpes virus UDG shares 39 and 49% homology with human and *E. coli* enzymes, respectively [55]. Human and *E. coli* UDGs share 73.3% homology [8, 61]. Generally, homology of amino acid

sequences of UDGs from various sources varies from 39 to 55.7% [61].

Mammalian cells are characterized by a high level of UDG gene transcription [61]. cDNA of human cell nuclear form UDG encodes a protein of 304 residues (33.8 kD). However, the purified protein looses 77 N-terminal residues [62, 63]. The human UDG encoding gene consists of six exons and five introns and it exhibits typical features of a housekeeper gene [64]. Nuclear and mitochondrial UDGs are formed by alternative splicing [65]. Point mutations in the third exon caused impairments in UDG structure and the development of glioblastomas [66]. Figure 3 shows the structure of human cell UDG gene.

The secondary structure of UDG contains alternations of eight α-helices and four β-sheets which form an ellipsoid of 52 × 30 × 20 Å [55]. A DNA-binding site is located in the C-domain; this site contains a cluster of a large number of highly conservative amino acid residues such as Asn, Gln, Asp, Phe, Gly, and Tyr which are involved in the recognition of dU in DNA and catalysis (see below) [55, 56]. Human and herpes simplex UDGs possess almost identical active structure [55, 56]. X-Ray analysis of *E. coli* UDG revealed that its structure (including residues forming the active site) is closer to the human enzyme rather than to the viral enzyme [67, 68].

**Cell localization and forms of UDG.** In many cells including KB cells, human lymphocytes, fibroblasts, placenta, and also rat liver nuclear and mitochondrial forms of UDG have been recognized [52, 69, 70]. The major proportion of UDG was found in nucleus and cytoplasm and only 5-15% of total cellular content of UDG was found in mitochondria [70]. These forms differ by chromatographic characteristics and other features. Molecular masses of nuclear and mitochondrial enzymes are 35-37 and 29 kD, respectively [45, 70]. Both forms exhibit distinct sensitivity to metal ions and thermostability. Mitochondrial enzyme is more resistant to heating: period of half-inactivation at 45°C for mitochondrial and nuclear UDGs is 18 and 4 min, respectively. Nuclear and mitochondrial enzymes also differ in sensitivity to sodium chloride; 25 mM NaCl inhibited nuclear UGD by 50% and activated the mitochondrial enzyme. The latter was inhibited by higher salt concentration (75 mM NaCl) [71].

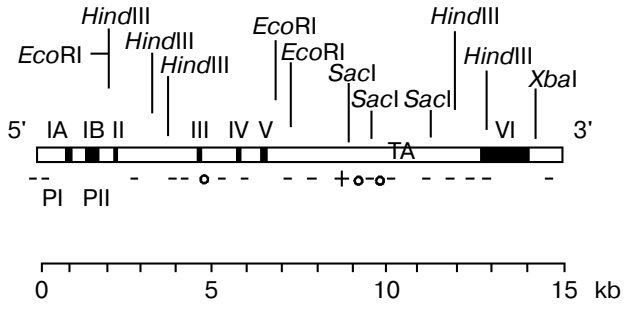


**Fig. 3.** Structure of gene encoding human uracil DNA glycosylase [64]. Exons are shown in black and numbers with Roman numerals. PI is promoter for exon IA (for transcription of nuclear form of UDG); PII is promoter for exon IB (for transcription of mitochondrial form of UDG). *Eco*RI, *Hind*III, *Sac*I, and *Xba*I indicate positions for corresponding restriction enzymes. *Alu*, *MER*, and *MIR* repeats are designated by (–), (o), and (+) respectively; dinucleotide repeat *TA* of 300 bp is designated as TA.

In contrast to the mitochondrial form, nuclear UDG exhibits temporal regulation which depends on the phase of the cell cycle. Interestingly, nuclear and mitochondrial forms of human UDG are encoded by the same gene [69] and these forms represent different variants of alternative splicing [72]. Nuclear and mitochondrial forms of human UDG contain signaling amino acid sequences required for particular (nuclear or mitochondrial) localization in the cell [73]. One of the nuclear isoforms (molecular mass of 36 kD) is phosphorylated at serine and threonine residues [74].

The nuclear form of UDG is associated with chromatin and possibly with DNA polymerase α [75]. Inhibition of UDG activity by monoclonal antibodies against DNA polymerase α reflects UDG interaction with the polymerase catalytic subunit (70 kD). It was also shown that UDG cDNA from human placenta is identical to cDNA encoding human glyceraldehyde-3-phosphate dehydrogenase subunit of 37 kD. The subunit of molecular mass 37 kD obtained from glyceraldehyde-3-phosphate dehydrogenase tetramer possesses all the properties of human placental UDG [76].

**Regulation of UDG activity during cell cycle.** There are changes in UDG activity which depend the phase of cell cycle and age of cell culture [77, 78]. The highest enzyme activity was observed at S-phase in young culture; on aging UDG activity is also detected in G1 and G2 phases [79]. Stimulation of lymphocytes with phytohemagglutinin was accompanied by ~10-fold increase of UDG activity [51]. During proliferation of synchronized fibroblast culture UDG activity increased ~5-fold just before the beginning of DNA replication [77]. UDG activity increased in S-phase and is maximal several hours before DNA synthesis [80]. Induction of UDG requires synthesis of RNA and proteins. However, experiments with inhibition of DNA replication with aphidicolin revealed that entry of fibroblast into S-phase is not the ultimate precondition for UDG accumulation [81]. It was also shown that the level of UDG activity is organ-specific and depends on the stage of the development of the organism. The highest level of mitochondrial UDG mRNA was found in skeletal muscles, heart, and sex glands, whereas maximal level of nuclear UDG mRNA was found in sex glands, placenta, thymus, and intestine

[82]. Enzyme activity may vary severalfold in organisms belonging to the same species [83-85]. Immunochemical methods revealed that in proliferating cells UDG is mainly localized in the nucleus and perinuclear space, whereas in resting cell the enzyme is mainly found in cytoplasm [77, 78, 80]. These changes have been found only for nuclear and cytoplasmic forms of UDG, whereas the mitochondrial enzyme remains at a constant level over the whole cell cycle [71].

Since mutant cells lacking all UDG activity have not been found yet, it remains unclear whether UDG is an enzyme vitally important for living cells. Since yeast and *E. coli* cells containing mutant UDG demonstrated normal development, it is possible that UDG is not an enzyme ultimately important for cell growth. However, it is possible that UDG activity is needed for normal development and differentiation of tissues of organisms [86, 87].

**Substrate specificity of UDG.** Various nucleic acids have been tested as possible substrates for UDG. Usually UDG can remove dU from single and double stranded DNA, the enzyme removing dU from single stranded DNA 2-3 times faster than from double stranded DNA [88]. However, calf thymus UDG is active with respect to double stranded DNA only [71]. RNAs containing UMP and dUMP as well as RNA–DNA duplex are not substrates for UDG [40]. The enzyme from *E. coli* removed 5′-terminal uracil only from phosphorylated 5′-terminus of the nucleotide chain [89]. The enzyme was unable to remove uracil from 3′-end of an oligonucleotide chain, but uracil residues could be readily removed from p(dU)p(dN)p oligonucleotide, which is the minimal substrate for UDG [89]. Excision of dU from oligonucleotide is especially effective when dU is located at the distance of 1-2 nucleosides before the 5′-end of the chain [89, 90].

Although UDG is inactive with respect to dUTP, dUMP, and 5-bromouracil, the enzyme may remove 5-fluorouracil (5-F-Ura) from 5-F-Ura-DNA and the $K_m$ value for 5-F-Ura-DNA (3.2 μM) is comparable to that of dU-DNA (0.2 μM) [91]. Lower affinity for 5-F-Ura-DNA suggests impaired binding of UDG with 5-F-Ura chain of DNA compared with dU; however, after enzyme–substrate complex formation the rate of 5-F-Ura excision was only 1.6 times lower than in the case of dU-DNA.

Reactive oxygen species (oxidative stress) may convert cytosine into products resembling uracil structure. Figure 4 shows structural analogs of uracil which are recognized and excised by human and *E. coli* UDG. However, it remains unclear whether UDG is involved in excision of damaged DNA bases appearing as the result of oxidative stress [92-94].

3,N4-Ethenocytosine formed in DNA during the action of such carcinogen as vinyl chloride or products of lipid peroxidation is recognized and excised by *E. coli*

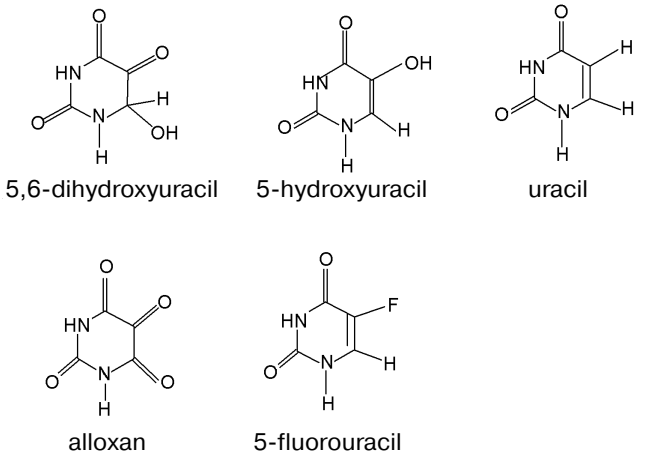


**Fig. 4.** Structures of uracil and its analogs which are excised from DNA by UDG.

UDG [95]. Human, viral, and bacterial UDG remove uracil from U : G base pairs of DNA more effectively than from U : A pairs [96, 97]. The rate of uracil excision catalyzed by *E. coli* UDG was two times higher in the case of U : G pairs than that of U : A pairs [98]. More effective recognition and excision of uracil from U : G pairs has evident biological sense because if a U : G base pair is not repaired before the next round of replication it may cause a transition whereas the U : A pair is not a mutagenic mutation. The latter may only impair binding of specific proteins to this particular site. However, in the cases of certain nucleotide sequences U is removed faster from U : A rather than from U : G pairs [98, 99].

The nucleotide environment around a modified chain of a DNA molecule significantly influences effectiveness of excision. For example, the rate of uracil excision is ten times higher for 5′-A/T A/T UA A/T A/T-3′ than for 5′-G/C G/C U G/C G/C-3′ or 5′-G/C G/C U TG/C G/C-3′ [96, 98, 99]. These data suggest that melting of DNA duplex is an important factor during uracil excision. In the case of single stranded oligonucleotide such dependence was not observed. Studies of phage M13 survival revealed that irrespectively to number of uracils located on one chain repair of uracil residues was more effective than repair of two uracils located on both chains not far from each other [100].

Treatment of DNA containing significant numbers of uracils (phage PBS 2 DNA, poly(dU,dC)) with UDG did not result in total uracil excision. It is possible that closely located adjacent uracils and apurinic sites formed during the enzyme action decrease catalytic activity of UDG. Apurinic sites were shown to inhibit calf thymus UDG [100]. X-Ray analysis and kinetic studies revealed that human UDG binds DNA with apurinic sites more effectively than that of dU-DNA [101].

Excision of uracils from DNA loops is more difficult [102]. Comparison of kinetic parameters of uracil excision from duplexes and loop structures revealed that in the case of loop structures the excision reaction was characterized by 5-fold higher $K_m$ and 3-4-times lower $V_{max}$ values than that for duplex. Addition of single stranded binding proteins (SSB-proteins) resulted in 7-140-fold increase of the effectiveness of uracil excision from the loop structures. However, in the case of single stranded DNA as substrate addition of SSB-proteins reduced effectiveness of uracil excision by 2-3-fold [103].

## MECHANISM OF DNA RECOGNITION AND EXCISION OF MODIFIED BASES BY URACIL DNA GLYCOSYLASE

**Interaction between UDG and nonspecific DNAs.** X-Ray analysis is one of the most informative approaches for study of enzymes. However, it does not allow quantitative evaluation of relative effectiveness of formation of certain contacts. So it is nearly impossible to take into consideration the contribution of specific and nonspecific interactions into affinity of enzymes for DNA. Ignoring this fact often leads to overestimation of the role of specific contacts between enzymes and DNA for affinity and specificity of enzyme action. For example, it is believed that $K_m$ values for modified DNAs reflect enzyme affinity to mutated bases [90, 104, 105]. These notions were further developed in studies of X-ray analysis of UDG–oligonucleotide complex [55, 56] and also during analysis of X-ray data for a representative number of other enzymes recognizing specific DNA [104, 105]. Some data on X-ray analysis of UDG have recently been summarized in reviews [6, 8]. Here we analyze data of X-ray analysis in light of results of thermodynamic and kinetic studies of UDG. We do believe that such an approach allows the evaluation of the actual contribution of certain contacts (recognized by X-ray analysis) into high affinity and specificity of enzyme action.

We found that interaction between enzymes and DNA can be effectively analyzed at the molecular level using gradual complication of DNA-ligand stretches (see for review [104, 105]). Using such an approach, we analyzed the mechanism of complex formation between human UDG and specific or nonspecific DNAs [90, 104-108]. Orthophosphate and any dNMP are minimal ligands for the UDG active site. These minimal ligands and oligonucleotides of any structure and length act as UDG inhibitors [90, 104-108]. Linearity of logarithmic dependencies of $K_d$ values on number ($1 \le n \le 10$) of mononucleotide chains of $d(pN)_n$ (Fig. 5) suggests additive interaction between UDG and 10-nucleotide chains of stretched single and double stranded DNAs [90, 104-108]. The protein globule of UDG covers 10 base pairs of DNA and each pair interacts with its subsite on UDG; a dU-recognizing subsite (active site) is located at the distance of 1-2 nucleotides from the 5′-end of the enzyme-bound decanucleotide (Fig. 6) [90].

Increase in the length of $d(pN)_{10}$ by one nucleotide ($n$ = 1-10) increased UDG affinity for this substrate by $f$ times in the following order: $d(pC)_n$ 1.47 < $d(pT)_n$ 1.65 < $d(pG)_n$ 1.82 < $d(pA)_n$ 1.9 (factor $f$ may be calculated using slopes given in Fig. 5 as an example) [90, 104-106]. Due to interaction with each internucleotide phosphate group affinity of UDG for single stranded DNA increases by 1.35 times (electrostatic factor $e$ = 1.35) [90]. Coefficients characterizing increase in UDG affinity for DNA due to hydrophobic or van der Waals interactions of the enzyme with one of the bases (hydrophobic factor $h_N$) were evaluated as $h_N = f/e$ and for $d(pC)_n$, $_d(pT)_n$, $d(pG)_n$, and $d(pA)_n$ this factor was 1.08, 1.22, 1.35, and 1.41, respectively. Experimentally obtained $K_d$ values for single stranded oligonucleotide of any sequence coincided with $K_d$ values calculated by means of diminishing geometric progression: $K_d[d(pN)_n] = K_d[(P_i)]\cdot(1.35)^{-n}\cdot(1.08)^{-l}\cdot(1.22)^{-m}\cdot(1.35)^{-k}\cdot(1.41)^{-g}$, where $l$, $m$, $k$, and $g$ are numbers of C, T, G, and A nucleotides in $d(pN)_n$ and $K_d[(P_i)]$ = 17 mM ($K_d$ value for orthophosphate) [90, 105].

Interestingly, this algorithm is general for description of any of the enzymes we studied [104, 105] and the only changes include values for $K_d[(P_i)]$ and factors $e$ and $h_N$ [105]. This algorithm is also applicable for description of the interaction of all studied enzymes not only with single

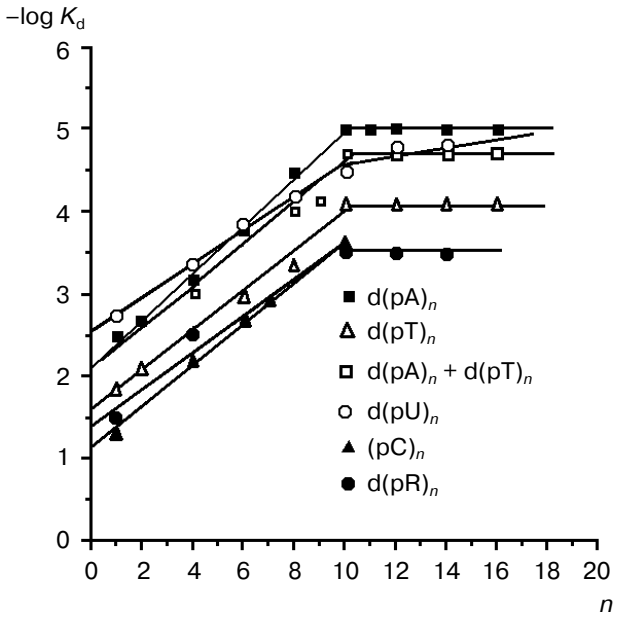


**Fig. 5.** Effect of ligand length ($n$) on negative logarithms of $K_d$ values for UDG complexes with various $d(pN)_n$ homooligonucleotides and their duplexes with complementary chains.

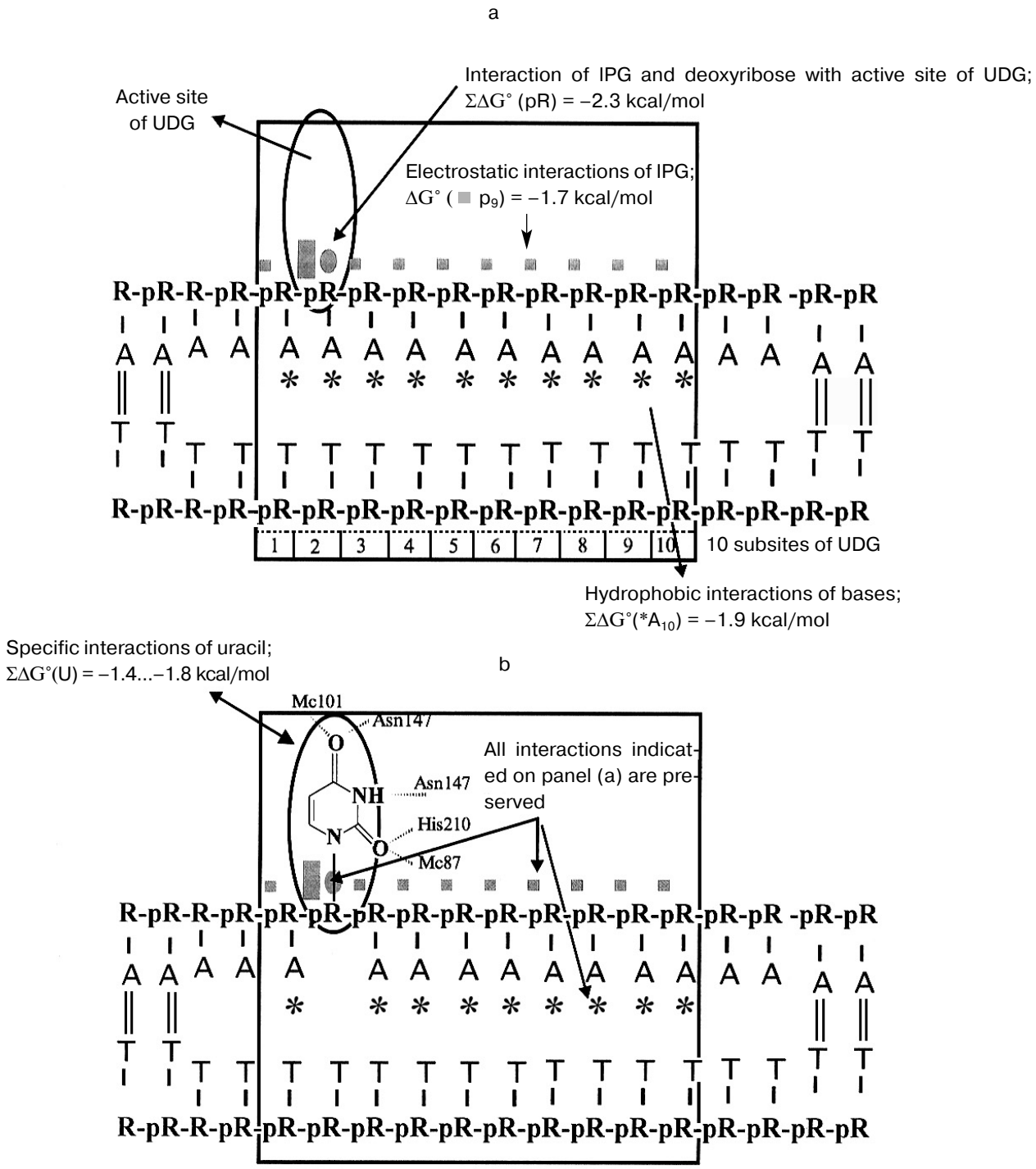


**Fig. 6.** Thermodynamic models for UDG interaction with nonspecific (a) and specific DNA (b) containing dU-unit in one of chanis: ■, weak electrostatic contacts of inter-nucleotide phosphate groups (IPG); *, weak hydrophobic and/or van der Waals contacts between enzyme and bases; ■, ● nonspecific contacts of d(pR) sugar phosphate residue of nonspecific dA- or specific dU-unit with the active of enzyme. Panel (b) shows specific hydrogen bonds formed by uracil with UDG amino acid residues recognized by X-ray analysis [55]; Mc101 and Mc87 correspond to N–H atoms of peptide bonds formed by Phe101 and Gln87; Asn147 forms a hydrogen bond with oxygen atom and NH-group of uracil due to $NH_2$ and C=O groups of side chain, respectively; His210 interacts with oxygen atom of uracil due to its NH-group of the imidazole moiety. The range of ΔG° changes for the dU-unit is given in dependence of nucleotide sequence of the substrate.

stranded but also with double stranded DNA. However, the value of the factor *f* characterizing the increase in the enzyme affinity on increasing length of oligonucleotide duplex by one base pair is only 1.1-1.5 times higher than in the case of single stranded DNA. Rather weak increase in the factor *f* during transition from single stranded to double stranded DNA is related to enzyme-dependent melting of the DNA stretch forming the complex with the enzyme. This attenuates complementary interactions between two chains compared with those determined for duplex in solution roughly by one order of magnitude. For DNA in solution a single base pair formation (A–T) is characterized by $\Delta G^\circ = -1.0$ to $-1.3$ kcal/mol; after binding to the enzyme this value decreased to $\Delta G^\circ = -0.3$ to $-0.4$ kcal/mol [104, 105].

According to data of X-ray analysis, the DNA-binding site of UDG is a groove 27 Å in length which is formed by positively charged amino acid residues [55, 56]. The diameter of this groove gradually narrows from 21 to 10 Å. The active site of UDG is located in the narrow part of the groove. We found that UDG may cause almost total melting of a 10-15 nucleotide stretch of DNA duplex [90]. This is consistent with data of X-ray analysis that both DNA chains interact with opposite sites of the DNA-binding channel [55, 56]. So within the protein globule of UDG the complementary chains of DNA almost do not interact with each other. The second chain of the DNA stretch is retained in the complex with the enzyme due to weak interactions with one of the "walls" of the DNA-binding channel and complementary interactions between chains outside the enzyme globule [90]. So the addition of the second chain does not increase UDG affinity for short duplexes compared with single stranded $d(pN)_{10\text{-}15}$ (Fig. 5). The second chain is obviously needed for formation of optimal conformation of the substrate (see below). Thus, UDG interaction with DNA duplex may be exhaustively described by the same algorithm as for single chain oligonucleotide (see above). On the basis of thermodynamic analysis of UDG interaction with DNA we concluded that the enzyme recognizes any single or double stranded DNA with reasonably high affinity ($K_d \sim 10$ μM) due to large number of weak additive electrostatic and hydrophobic and/or van der Waals interactions of the enzyme with separate nucleotides of the ligand [90, 106-110]. Values of $\Delta G^\circ$ characterizing electrostatic interactions between UDG and DNA are rather small ($-0.2$ to $-0.3$ kcal/mol) and comparable with those characterizing dipole–dipole and ion–dipole interactions. Consequently, interaction between negatively charged phosphate groups of DNA and positively charged zones on the UDG surface resembles interaction of biopolymer surfaces of opposite charges rather than direct interactions of contacting groups. This conclusion is supported by data of X-ray analysis of UDG which suggest the existence of a large number of positively charged amino acid residues in the DNA-binding channel [55, 56]. So it is reasonable to suggest that UDG should effectively interact with any negatively charged biopolymers. Infection of *B. subtilis* cells with bacteriophages PBS 1 and PBS 2 results in expression of phage protein, which inactivates host UDG protecting its genomic DNA. This protein consists of 84 amino acid residues and its surface is formed by negatively charged residues [111-114]. The protein inhibits UDG from various sources without any effect on the other related enzymes such as 3-methyladenine-DNA-glycosylase and hypoxanthine-DNA-glycosylase [111, 112, 115]. The protein inhibitor does not interact with active site of UDG directly, but simulating DNA duplex by shape and charge this protein interacts with the DNA-binding groove of UDG [114, 115]. Uncharged (ethylated on phosphate groups) oligonucleotides do not interact with UDG [90]. The affinity of UDG for 21-membered oligonucleotide containing uracil residues and in which sugar-phosphate backbone was substituted for uncharged polypeptide chain containing nucleoamino acid residues *L-3-(uracil-1)alanine* is very low; it is comparable with the affinity of UDG for $d[Tp(Et)]_{14}$ [108, 109]. This also confirms the importance of nonspecific electrostatic interactions between UDG and substrate required for high enzyme affinity for DNA.

All these data have been summarized using a thermodynamic model (Fig. 6a) describing UDG interaction with $d(pA)_{10}{\cdot}d(pT)_{10}$ duplex. This model shows the relative contribution of separate structural elements of the duplex underlying high enzyme affinity: $\Delta G^\circ$ value characterizing active site interaction with one of deoxyribonucleotide residues $d(pA)_{10}$ of $\approx -2.3$ kcal/mol, $\Delta G^\circ$ value characterizing electrostatic interactions of nine internucleotide phosphate groups of $\approx -1.7$ kcal/mol; $\Delta G^\circ$ characterizing hydrophobic interactions of ten A-bases of $d(pA)_{10}$ of $\approx -1.9$ kcal/mol. So, nonspecific interactions between DNA and UDG provide about five orders of affinity ($\Delta G^\circ \approx -5.9$ kcal/mol).

Using dU plasmids and labeled concatomers, it was shown that rat UDG moves along DNA until it "meets" uracil, i.e., the enzyme possesses a processive mechanism for searching of the damaged base [116, 117]. Increase of NaCl concentration causes transition from processive to distributive mechanism [116, 117]. Consequently, high affinity of UDG to nonspecific DNA allows this enzyme to bind to any DNA sequence and to "slide" along DNA in the search of dU. Such mechanism provides high rate of uracil excision.

**UDG interaction with specific DNAs.** UDG has ten times higher affinity for dUMP and $d(pU)_n$ than to any dNMP and nonspecific oligonucleotides of various lengths and sequences [90, 107, 110]. Insertion of dU into various oligonucleotide duplexes resulted in 9-20-fold increase in the enzyme affinity for these substrates (compared with substrates lacking dU). Increase in the enzyme affinity with respect to specific dU-oligonucleotides also depended on the oligonucleotide sequence and the

opposing base at the second strand. In the case of non-complement pairs U–C and U–G enzyme affinity for duplexes was 11-16 times higher than for control duplex lacking dU. In the case non-complement pair U–T the enzyme affinity was only 4 times higher compared with corresponding control. Interestingly, contribution of dU in UDG affinity for DNA did not differ in the case of stretched DNA and free dUMP [90]. Consequently, interaction of UDG with specific and nonspecific DNAs approaches additivity. According to our data, the contribution of all specific interaction of dU in the resultant affinity of UDG for DNA does not exceed one order of magnitude ($\Delta G^\circ \approx -1.4$ to $-1.8$ kcal/mol) whereas nonspecific contacts provide about 5 orders of magnitude ($\Delta G^\circ \approx -5.9$ kcal/mol). Thermodynamic analysis of DNA binding and uracil "twisting" by means of non-hydrolyzed substrate analogs (2′-α and 2′-β isomers of 2′-fluoro-2′-deoxyribouridine) confirmed our data that uracil "twisting" and its interaction with the enzyme are characterized by $\Delta G^\circ < -2$ kcal/mol [118]. This suggests that recognition of dU by UDG has minor contribution to effectiveness of enzyme–DNA complex formation and therefore it cannot provide specificity of the enzyme action. Moreover, effectiveness of UDG recognition of partially non-complementary complexes lacking dU is comparable to that of found for dU-DNA. Values of $K_d$ for duplexes $d(pA)_{15}\cdot d(pT)_7(pC)(pT)_7$ and $(pA)_{15}\cdot d(pT)_7(pG)(pT)_7$ were 2.5 and 5.4 times lower than for $d(pA)_{15}\cdot d(pT)_{15}$. Consequently, UDG may effectively recognize non-complement base pairs lacking uracil; the difference in enzyme recognition of non-complement pairs U–C, U–G, U–T and also for A–C, A–G did not exceed 2-8 times [90, 107]. Increase in enzyme affinity for DNA due to the presence of dU we described by the thermodynamic model (Fig. 6b), which shows that all specific interactions between UDG and dU of DNA are characterized by $\Delta G^\circ \approx -1.4$ to $-1.8$ kcal/mol (~1 affinity order) [90].

Other sequence-specific enzymes are also characterized by one order of magnitude increase in their affinity for specific DNAs: maximal increase in affinity due to interaction with specific sequence varies from 100 to 250 times [105]. Consequently, the thermodynamic stage, complex formation, cannot provide specificity of these enzymes including UDG [90, 104, 105]. Nevertheless, they are known as sequence-specific. So we evaluated the rate of the reaction catalyzed by UDG in the presence of various DNA substrates; transition from nonspecific to specific DNAs was accompanied by reduction of the reaction rate by 4-5 orders of magnitude [90]. Even minimal modifications of a base or sugar residue of deoxyuridine unit of the oligonucleotide used significantly reduced the rate of uracil excision up to total arrest of this reaction [108, 109].

Using UDG and also other enzymes of replication, repair, topoisomerization, restriction, and integration, it was shown that stages of enzyme-dependent adaptation of DNA to optimal conformation and catalysis are the basis of specificity of enzyme action. Transition from nonspecific to specific DNAs resulted in the increase in $k_{cat}$ value by 4-8 orders of magnitude [104, 105]. Below we consider how DNA binding to UDG influences its conformation required for high reaction rate.

**Conformational adaptation of DNA and UDG.** For each DNA-dependent enzyme optimal structure of DNA has distinct characteristic features determined by distribution of hydrophobic, hydrophilic, and charged zone in the DNA binding site [104, 105]. After adaptation of DNA structure to optimal conformation, which depends on initial DNA structure in solution and its ability to change conformation in the enzyme-driven direction, catalytic conversion of substrate occurs [104, 105]. Our experimental data and results of X-ray analysis of UDG suggest that a similar situation also takes place in the case of UDG [90].

The narrow end of the DNA-binding channel of UDG is a U-shaped loop formed by Pro residues; channel margins are also formed by loops containing many residues of "rigid" Pro [55, 56]. These loops form contacts with other elements of secondary structure of the protein. Rigidity of active site structure of UDG implies the necessity of certain conformational rearrangements of both DNA and UDG during the stage of enzyme–DNA complex formation.

X-Ray analysis of UDG complexes with uracil and $d(pT)_3$ followed by computer modeling of enzyme interaction with DNA stretches revealed that UDG binding to DNA caused significant changes in the sugar-phosphate backbone and the resultant conformation is not typical for B-form DNA in solution [55, 56]. Conservative Leu272 located just above the active site of human UDG is suggested to be involved into local melting of DNA helix, by intercalating its hydrophobic side chain between bases [56]. Subsequent enzyme-induced change of DNA structure include its melting, damage to stacking interactions of double helix, followed by subsequent inside out "twisting" of a uracil residue. After that the uracil residue enters the uracil-binding pocket of UDG. Catalysis of the uracil excision reaction becomes possible after additional structural rearrangements of the dU unit of DNA in the active site. A similar scenario is also realized in the case of Hha1 (cytosine-5)-methyl transferase [119].

Adaptation of single and double stranded DNAs depends on their structure in solution [90, 107, 108]. For example, UDG is characterized by higher affinity to single stranded homo-$d(pN)_n$ than to corresponding duplexes. However, this enzyme exhibited 4-50 times lower affinity to single stranded homo-$d[(pN)_n]$ containing one heterogeneous unit like $d[(pT)_n(pU)(pT)_m]$ or $d[(pA)_n(pU)(pA)_m]$ than to corresponding homooligonucleotides. The effectiveness of complex formation between UDG and such oligonucleotides did not depend

on the type of heterogeneous base. For example, UDG exhibited nearly the same affinity for both d[$(pT)_n(pU)(pT)_m$] and oligothymidylates containing dC or dG instead of dU. However, transition from homo- to hetero-dU-oligonucleotides and from single to double stranded ligands containing dU unit such "abnormal" interaction with enzyme disappeared. Consequently, effectiveness of mutual adaptation of UDG and DNA depends on primary structure of single stranded DNA. Absence of abnormalities in duplexes suggests that the second complementary chain of DNA plays an active role in formation of optimal conformation of UDG substrate [90, 107, 108].

It should be mentioned that the active site of UDG exhibits strict requirements for initial structure of nucleotide chain of the ligand than other 9 of 10 subsites (Fig. 6), recognizing nucleotide units of DNA molecules at the stage of complex formation and during catalysis. For example, UDG affinity for various ribo-$(pN)_n$ is one order of magnitude lower than that for corresponding deoxy-oligonucleotides of the same length [90, 107]. However, reduction in the enzyme affinity is related only to reduction (by one order of magnitude) of effectiveness of interaction of uracil-binding site with one unit of ribooligonucleotide. Other 9 units of ribo-$(pN)_{10}$ interacted with the enzyme with the same effectiveness as $d(pN)_{10}$. A similar phenomenon was observed during UDG interaction with RNA–RNA and RNA–DNA duplexes. During transition from DNA–DNA complexes to RNA–RNA or mixed DNA–RNA complexes the enzyme affinity reduced by 5-12 times [90, 105]. These differences in the interaction of UDG with duplexes may be explained by differences in the structure of DNA–DNA, DNA–RNA, and RNA–RNA complexes. UDG exhibits greater effectiveness in the interaction with duplexes existing as B-forms in solution [90]. According to literature data the duplex $(pA)_n \cdot d(pT)_n$ is that RNA–DNA duplex which may exist in B-form [120]. The enzyme exhibits similar affinity to this duplex and to double stranded deoxy-oligonucleotide, whereas affinity to other mixed duplexes is lower. Thus, UDG selects substrates at the stage of complex formation, but this stage cannot provide highly effective discrimination of single or double stranded (deoxy)ribo-oligonucleotides by this enzyme.

We found that UDG excises bases from dA-, dT-, dG-, and dC-units of DNA; however, the rate of these reactions is 4-5 orders of magnitude less than in the case of dU-unit [90, 105]. In spite of close affinity of UDG for ribo- and deoxyribo-ligands this enzymes cannot excise uracil from RNA or DNA containing a riboU-unit even at saturating concentration [90, 105]. This suggests that UDG cannot alter the structure of a riboU-unit to the conformation required for catalysis. Other (even minimal) modifications of sugar residue of dU-unit also strongly reduced the rate of reaction catalyzed by UDG. For example, UDG affinity for double stranded oligonucleotide containing 2′-amino-2′-deoxyuridine was higher than for control deoxyuridine containing oligonucleotide [106, 107]. However, no uracil excision was observed. Insertion of a dU residue containing fluoride in the 2′-position of sugar resulted in appearance of the double stranded oligonucleotide unit in A-conformation (80% 2′-fluoronucleosides are in 3′-*endo*-conformation). UDG recognizes such conformational motif ten times worse than the control oligonucleotide and uracil excision was almost absent [108, 109].

According to X-ray analysis among all natural dNMP only dUMP may exist in two thermodynamically equivalent conformations, only one of which promotes base stacking in a DNA molecule [121]. Easy transition of a dU-unit from C1′-*exo*-conformation to C2′-*endo*-conformation during maintenance of base *anti*-conformation, required for effective catalysis, should promote dU-DNA melting by UDG. This facilitates inside out twisting of uracil from double stranded DNA and increases the rate of reaction catalyzed by UDG. *Trans-gauche* conformation for C4′–C5′-bond of 5′-dUMP unit is more optimal for catalysis than *gauche-gauche* conformation of this nucleotide, which is typical for double stranded DNA [90].

In some trimers like purine-pyrimidine-purine, pyrimidine "protrudes" from the trimer and two purines form stacking contacts. This effect of "adjacent neighbors" is especially demonstrative when uracil is used as the pyrimidine. A similar situation is probably observed in stretched oligonucleotides [122]. Reduction of the rate of uracil excision is generally observed during transition from single to double stranded oligonucleotide and then to "hairpin"-containing substrate and G–C-rich duplexes [108, 109]. This also confirms the conclusion that easy melting of DNA duplex is an important factor predetermining the rate of uracil removal.

Thus, effectiveness of substrate DNA fit to conformation required for enzymatic catalysis depends on many factors which we have considered. However, the catalytic stage is more sensitive to DNA structure than the stage of complex formation between UDG and DNA ligand [110].

**Additional factors underlying specificity of UDG action.** Stages of conformational changes of DNA result in stabilization of UDG complex with dU-containing sites of DNA and primary selection of substrate. However, at the subsequent stages of catalysis of base excision there are several additional ways for discrimination of "right" and "wrong" DNA bases by UDG. One of such ways includes five pseudo Watson–Crick hydrogen bonds of the dU-unit of DNA with Asn147, Gln87, Asp88, Phe101 residues of herpes simplex UDG (Fig. 6) recognized by X-ray analysis [55].

In the presence of enzyme increase of the reaction rate by 6-12 orders compared with the same (but nonen-

zymatic) reaction in solution is determined by nine various factors like decrease of activation energy, increase of effective substrate concentration, etc. [123]. Phenomenon or "orbital driving", i.e., orbital fit of reacting groups of enzyme and substrate with accuracy up to 10-15° is the other important factor responsible for increase of enzymatic reaction rate [123]. Tension determined by bond deformation by 10° is only 2.7 kcal/mol and such energetic barrier can be easily overcome at 25-30°C.

After protrusion of uracil from the stacking deoxyribose and 5′-phosphate "turn" about 180° and shift by 15 Å and the interaction of Leu272 with DNA stabilized such conformation [56]. Only uracil can freely penetrate into the UDG active site and form hydrogen bonds with side chain of Asn147 and functional groups Gln87, Asp88, and Phe101 (Fig. 6) [55, 56]. Uracil forms stacking and van der Waals contacts with the phenyl ring of Tyr90, and N1 of uracil forms hydrogen bonds with solvent molecules. This determines the orientation of the dU-unit in the active site of UDG [55]. X-Ray analysis revealed that human UDG forms hydrogen bonds with O2, N3, and O4 atoms of uracil (Fig. 6) and this allows distinguishing uracil from cytosine, thymine, and purine bases [56]. The same contacts have also been found in the vase of herpes simplex UDG [55]. Thus, formation of pseudo Watson–Crick hydrogen bonds with uracil should promote specified orientation of electronic orbitals of glycosidic bond of uracil and water molecules in the enzyme pocket; this is required for maximal overlapping of corresponding orbitals at the transition state and, consequently, for increase of uracil excision rate by 4-6 orders of magnitude.

Results of X-ray analysis of UDG–DNA complex suggest that the "orbital driving" of the reaction and "under-fit" of interacting orbitals in the case of various nonspecific nucleotide units of DNA may be achieved in various ways. For example, cytosine having the same size as uracil may penetrate into the active site of UDG but it cannot form specific hydrogen bonds required for catalysis [55, 56]. Adenine and guanine are too large and they cannot effectively penetrate into the active site as uracil, because the imidazole moiety of the purine ring will "collide" with the side chain of Tyr90 and form unfavorable contacts preventing purine penetration into the active site. (This was demonstrated by X-ray analysis and computer modeling). Thymine might penetrate into the active site and form "correct" hydrogen bonds with the enzyme, but, according to X-ray analysis, the 5-methyl group will be positioned in the uracil binding pocket and occupy the position of water involved in hydrolysis of N-glycosidic bond of the dU-unit [55, 56]. It is also suggested that the entrance to the active site of UDG contains a thymine alternative binding site, which may operate as scavenger preventing potential productive binding of thymine at the active site. All these possible reasons explaining reduction of the reaction rate in the case of nonspecific dN-units of DNA were validated by using mutant forms of UDG [124, 125]. Substitution of Asn204 (of human UDG) for Asp resulted in appearance of very weak but detectable cytosine-DNA-glycosylase activity. Substitution of Tyr147 for smaller sized Ala, Ser, or Cys led to appearance of weak thymine-DNA-glycosylase activity.

Since UDG can catalyze low effective base excision from nonspecific dN-units of DNA it is clear that the multistage selection of the "right" base for excision is not absolutely correct. UDG is able (with different efficacy) to melt DNA duplexes lacking dU-units and nonspecific bases can penetrate into the active site of the enzyme. Factors responsible for substrate selection by the enzyme mainly operate at the stage of substrate fit to optimal conformation, "orbital driving", and catalytic stage but at the stage of complex formation between UDG and DNA [90]. Moreover, according to data of thermodynamic analysis, even if nucleotide base is not "twisted out" from the DNA structure its phosphate group and sugar residue effectively interact with the active site of UDG and contribute to two affinity orders ($K_d$ for a nonspecific unit is about 17-33 mM, see Fig. 6a) whereas effectiveness of enzyme–base interaction does not exceed one order [90].

According to X-ray analysis and computer modeling the side chain of the conservative Gln144 residue apparently initiates and stabilizes such conformation of dU-unit at the active site of UDG when the C-1′ atom of deoxyribose is susceptible for nucleophilic attack by a water molecule. We already mentioned above that modifications of sugar residues of dU-unit mainly influence the rate of enzymatic reaction. The additional 2′-OH group of ribose of RNA-ligands is believed to prevent sterically such nucleophilic attack and, consequently, enzymatic catalysis [55, 56]. Thus, UDG discrimination between DNA and RNA duplexes is mainly realized at the stage of catalysis [90, 104-108].

## CATALYTIC MECHANISM

On the basis of X-ray analyses of UDG from herpes simplex, human fibroblasts, and *E. coli* cells several possible mechanisms of hydrolysis of N-glycosidic bond have been proposed [55, 56]. The stage of complex formation begins with Leu272 intercalation into the minor groove of DNA. Simultaneously Leu "pushes" uracil out of the DNA duplex and facilitates formation of necessary enzyme–substrate contacts. Contacts between protein and sugar-phosphate backbone strongly affect the backbone conformation and cause DNA compression. This results in uracil pushing outside DNA duplex into the active site of the enzyme, where uracil binding and polarization of the N-glycosidic bond occur due to enzyme–uracil hydrogen bond formation [55, 56].

Specific contacts between UDG and dU-unit increase enzyme affinity for DNA by one order of magni-

tude ($\Delta G^\circ \approx -1.8$ kcal/mol, see Fig. 6). Five hydrogen bonds formed between uracil and UDG (Fig. 6) significantly contribute to stabilization of the transition state and realization of the "orbital driving" mechanism [90, 105].

**Catalytic mechanism of herpes simplex UDG.** According to X-ray analysis herpes simplex UDG [55] and a water molecule activated by Asp88 (Asp145 in human UDG) attack the C1′ atom of deoxyribose (Fig. 7). Asp acts as a base attracting a proton of the water molecule. Conservative behavior of Asp145 as well as almost total loss of catalytic activity after Asp substitution for Glu underlines the crucial role of the side chain of Asp in catalysis. Nevertheless, it seems unlikely that water attack is sufficient for N-glycosidic bond cleavage; obviously, some additional factors weakening this bond are needed. Protonation of uracil aromatic ring might act as such factor. Although direct protonation of N-1 seems to be unlikely because its electrons are involved in the π-system of the ring, this effect may be achieved by protonating O-2. In contrast to other groups O-2 oxygen atom is relatively opened and it is involved only in formation of a single hydrogen bond. Although O-2 is positioned towards His210 (His268 in human UDG), a source of protons, the distance between nitrogen of histidine ring and O-2 of 5.1 Å is too long for direct proton transfer. Oligonucleotide uracil is effectively removed only when at least two phosphate groups from the 3′-end (including dU-unit) exist. It is possible that these phosphate groups collide with histidine. This causes its shift of 2 Å followed by O-2 protonation of dU-unit by histidine and increase of the rate of hydrolysis of N-glycosidic bond [55].

**Catalytic mechanism of human UDG.** The catalytic mechanism proposed for human UDG suggested that in the reaction of nucleophilic substitution His268 attacks the C1′ atom of the furanose ring either directly or by accepting a proton from $H_2O$ [56]. Amino acid residues Asp145, Asn204, and Gln144 form hydrogen bonds with O2, N3, and O4 atoms of uracil. This not only provides "correct" nucleotide orientation at the active site of the enzyme but also stabilizes its anionic state by polarizing the glycosidic bond and facilitating the nucleophilic attack. Amide groups of Asp145 and Gln144 form a "pocket" for negatively charged O2 atom of uracil [56]. Figure 8 gives schematic presentation of the reaction mechanism proposed by Mol et al. [56].

However, the pH dependence of UDG activity questions a catalytic mechanism involving direct protonation of the O2 atom of uracil by His268 [122]. Since His268 is localized on the surface of the enzyme, this residue should have $pK_a$ value corresponding (or close to) the $pK_a$ value of free histidine (i.e., $pK_a \sim 6.5$). However, UDG retains about 75% of its catalytic activity at pH 10. Under these conditions it seems unlikely that His268 is protonated. It was shown that in free enzyme His268 is neutral; however, during enzyme–substrate complex formation the $pK_a$ value of the histidine residue is about 7.1 due to binding of a water molecule and neighboring negatively charged DNA. Thus, His268 cannot act as a positively charged group. Evidently, His activates a water molecule by attracting negatively charged hydroxide ion. In the "enzyme–product" complex the $pK_a$ value for His268 becomes neutral again [122]. Recent structural studies of UDG complex with dU-DNA revealed that due to its orientation His268 cannot exert direct nucleophilic attack on C1′-atom of deoxyribose or serve as the catalytic base as suggested earlier [101, 126].

Asp145 is the other important amino acid involved in catalysis. It is suggested that Asp145 acts as the catalytic base attracting a proton from a water molecule and forming nucleophilic hydroxyl [122]. Effectiveness of such process requires $pK_a$ value for Asp145 to be higher than for free Asp in solution. In free enzyme the $pK_a$ value for Asp145 is about 4.1; this is close to the $pK_a$ value for free Asp. In enzyme–substrate complex the $pK_a$ value for Asp increases to 4.4 due to the presence of negatively charged DNA molecule at the active site. However, since the carboxyl group of this residue is located at the surface of UDG there is insignificant increase in $pK_a$ value. In enzyme–product complex the $pK_a$ value for Asp145 is reduced to ~3.0 due to Asp contact with Lys218 located on the adjacent loop. Since the $pK_a$ value for Asp145 is low the latter cannot act as the catalytic base and induce hydroxide-ion formation. Moreover, close positioning of negatively charged 3′-phosphate also prevents proton transfer from water. In contrast to Mon et al. [56], Luo et al. [122] proposed a catalytic mechanism (Fig. 8) that employs two water molecules bound to Asp145 and 3′-phosphate in proton transfer. Proton transfer from one water molecule to another results in formation of hydroxide-ion and hydroxonium ion. The latter stabilizes close positioning of negatively charged 3′-phosphate and Asp145, whereas hydroxide-ion acts as nucleophile. Proton of His268 activates a water molecule and its proton attacks the O2 atom of uracil.

**Catalytic mechanism of *E. coli* UDG.** A catalytic mechanism for *E. coli* UDG was proposed on the basis of X-ray analysis and site-directed mutagenesis. This mechanism implies polarization of N1–C1′ glycosidic bond due to attraction of electron density of the C1′-atom of deoxyribose through N1 to the uracil moiety [55-57]. Redistribution of electron density is accompanied by formation of two resonant structures stabilized by hydrogen bonds between the base and side chains of His187 and Asn123 and also Gln163 of the main chain. A water molecule activated by Asp64 attacks the C1′-atom of deoxyribose. In contrast to earlier hypotheses [55, 56], Svendsen et al. [57] suggest that protonation of the O2 atom of uracil is not essential because redistribution of negative charge (which characterizes the transition state) involved N1, O2, and O4 atoms of uracil. Being donor of neutral hydrogen bond for O2-uracil atom neutral His187 stabilizes the tran-

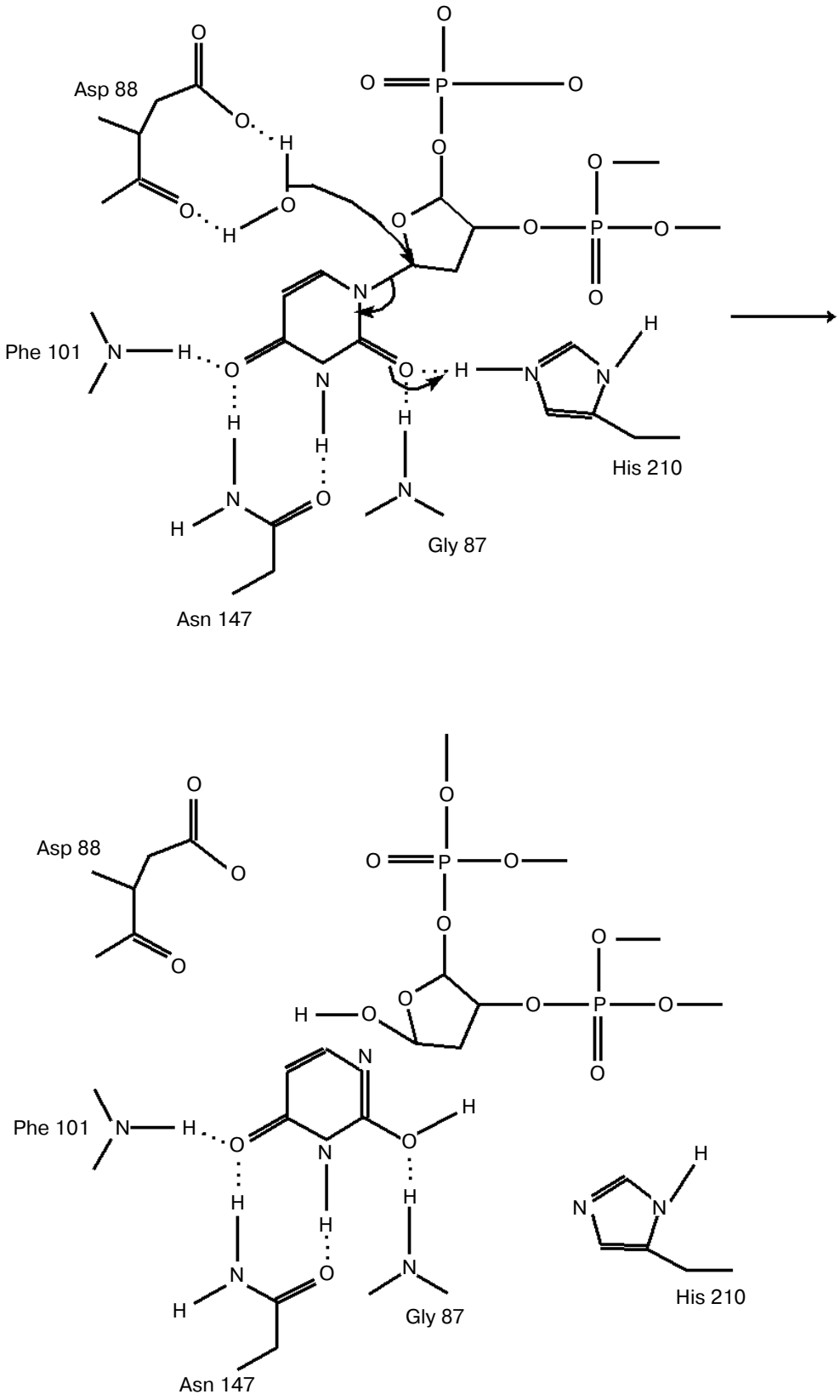


**Fig. 7.** Schematic presentation of the reaction mechanism of uracil removal from the dU-unit of DNA catalyzed by herpes simplex UDG [55].

sition state [57, 127]. It is suggested that Asp64 and His187 contribute to stabilization of "twisted out" uracil with $\Delta G^{\circ}$ less than –0.4 kcal/mol. Substitution of His187 for Gln or Ala destabilizes the transition state. The authors suggest that these residues weakly interact with substrate and product, but strongly interact with transition state [57].

Data obtained employing various independent methods suggest that there are common principles in

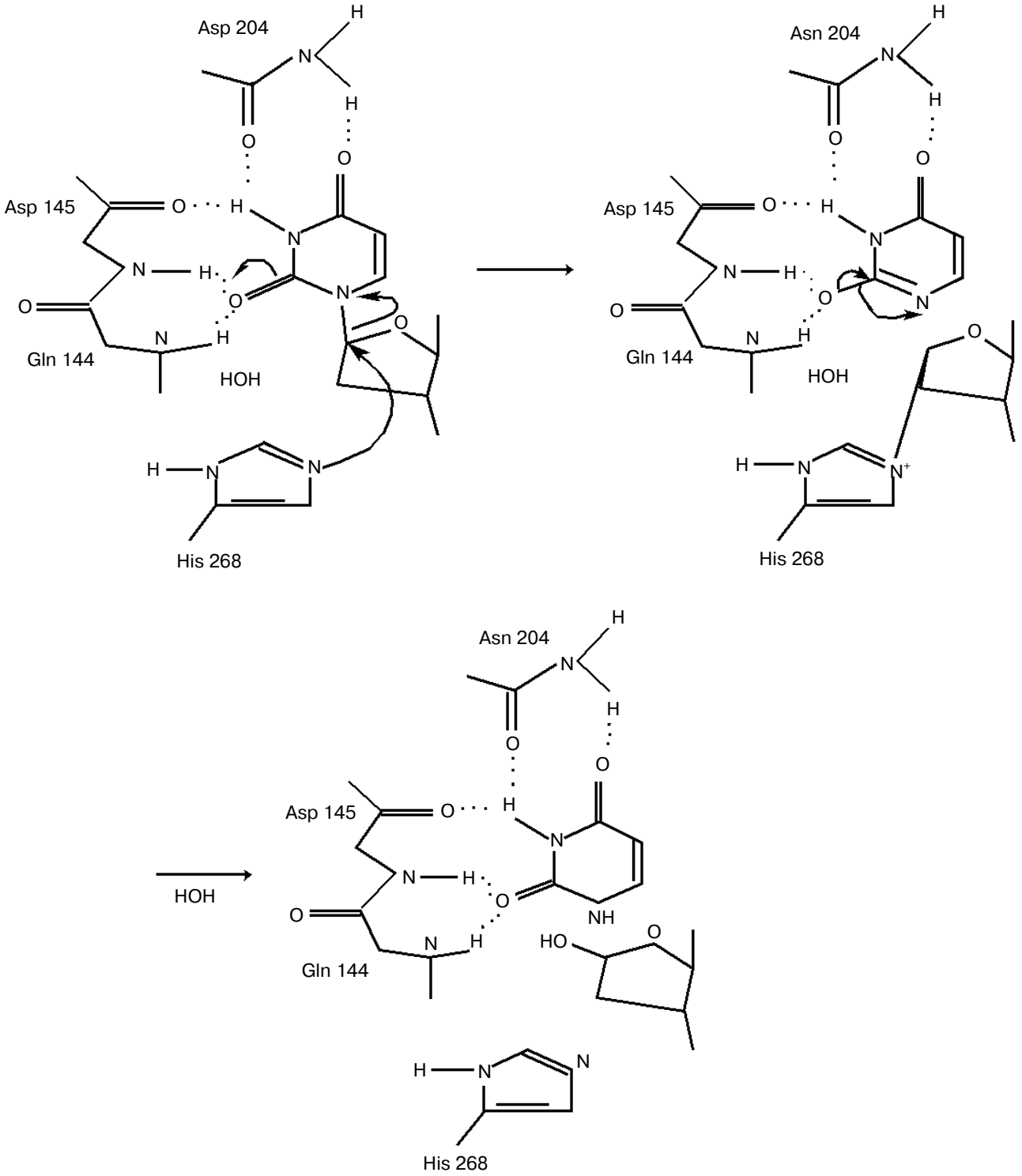


**Fig. 8.** Schematic presentation of the reaction mechanism of uracil removal from the dU-unit of DNA catalyzed by human UDG [56].

functioning of UDG from various sources including viruses and pro- and eukaryotes. Like many other enzymes, UDG exhibits high affinity for "heterogeneous" DNAs [100, 104, 105]. The interpretation of this phenomenon often employs a popular notion that sequence-specific enzymes can effectively recognize separate nucleotide units or blocks of "heterogeneous" DNAs containing elements of specific sequences. As shown above, UDG interaction with any DNAs is nearly the same as in the case of dU-DNA, but enzyme-driven "maximally effective change of substrate structure" occurs only with dU-DNA. High affinity of UDG for any DNA sequence provides the possibility for enzyme "sliding" along DNA which is important for fast search of dU-units.

Enzymes are known to be complementary to transition state realized during catalytic conversion of substrate rather than to substrate. In this connection it is reasonable to suggest that our previous evaluation of UDG affinity for various DNA and RNA ligands [90, 104-108] did not take into consideration possible increase of effectiveness of complex formation at the stage of the transition state. However, effectiveness of dU-containing substrates selection by UDG determined by the stage of complex formation (~1 affinity order) and subsequent stages underlying changes of DNA conformation and catalysis (increase in the rate by 4-5 orders of magnitude) finally give 5-6 orders of UDG specificity. This is consistent with UDG specificity observed *in vivo*. Thus, good evidence exists that the basis of specificity and functioning of UDG is determined not by the stage of complex formation but subsequent stages which (depending on substrate structure) finally determine the relative value of the rate constant ($k_{cat}$) of the N-glycosylase reaction.

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