THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 1, Issue of January 2, pp. 45–50, 1998

Printed in U.S.A.

Direct Measurement of the Substrate Preference of Uracil-DNA Glycosylase*

(Received for publication, June 12, 1997, and in revised form, September 21, 1997)

George Panayotou‡, Tom Brown§, Tom Barlow§, Laurence H. Pearl¶‖, and Renos Savva¶**

From the ‡Ludwig Institute for Cancer Research, University College London, 91 Riding House Street, London, W1P 8BT, the §Department of Chemistry, University of Southampton, Highfield, Southampton, SO17 1BJ, and the ¶Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, United Kingdom

Site-directed mutants of the herpes simplex virus type 1 uracil-DNA glycosylase lacking catalytic activity have been used to probe the substrate recognition of this highly conserved and ubiquitous class of DNA-repair enzyme utilizing surface plasmon resonance. The residues aspartic acid-88 and histidine-210, implicated in the catalytic mechanism of the enzyme (Savva, R., McAuley-Hecht, K., Brown, T., and Pearl, L. (1995) *Nature* 373, 487–493; Slupphaug, G., Mol, C. D., Kavli, B., Arvai, A. S., Krokan, H. E. and Tainer, J. A. (1996) *Nature* 384, 87–92) were separately mutated to asparagine to allow investigations of substrate recognition in the absence of catalysis. The mutants were shown to be correctly folded and to lack catalytic activity. Binding to single- and double-stranded oligonucleotides, with or without uracil, was monitored by real-time biomolecular interaction analysis using surface plasmon resonance. Both mutants exhibited comparable rates of binding and dissociation on the same uracil-containing substrates. Interaction with single-stranded uracil-DNA was found to be stronger than with double-stranded uracil-DNA, and the binding to Gua:Ura mismatches was significantly stronger than that to Ade:Ura base pairs suggesting that the stability of the base pair determines the efficiency of interaction. Also, there was negligible interaction between the mutants and single- or double-stranded DNA lacking uracil, or with DNA containing abasic sites. These results suggest that it is uracil in the DNA, rather than DNA itself, that is recognized by the uracil-DNA glycosylases.

Uracil-DNA glycosylases (UDG)[1] are a highly conserved and ubiquitous class of DNA-repair enzymes that catalyze the excision of uracil bases from DNA (1). Uracil is an RNA base and does not normally occur in DNA, though it forms a good Watson-Crick base pair with adenine. Uracil and thymine differ in that thymine is methylated at the 5-position of the pyrimidine ring, and this is not involved in forming the base pair. Uracil can occur in DNA either by misincorporation of deoxyuridine triphosphate during replication by DNA polymerases, which apparently do not discriminate between this nucleotide and thymidine triphosphate, or as the product of spontaneous hydrolytic deamination of cytosine residues in DNA (1, 2). The Gua:Ura mismatches that result from cytosine deamination are promutagenic, leading to Gua:Cyt → Ade:Thy transition mutations unless the uracil is repaired to the original cytosine (1, 2). As thymine methyls in Ade:Thy base pairs are essential in sequence-specific recognition by many regulatory DNA-binding proteins (3), Ade:Ura base pairs, although not mutagenic, are potentially disruptive.

Recently, the structural basis for the exquisite recognition of uracil by UDGs has been elucidated (4–6). The structures show that these enzymes are able to accomodate sequence nonspecific DNA along a channel and that this channel contains an active site pocket that is perfectly tailored to admit only uracil bases. The evidence for a DNA-binding channel is further supported by the structures of a specific peptide inhibitor of the UDGs in complex with the enzymes. This inhibitor prevents binding of UDG to DNA, and is seen to mimic a polynucleotide in its interaction with the enzyme (7, 8).

The structures of DNA modification and repair enzymes in complex with polynucleotides either show directly, or indicate the likelihood of, the extrusion of the target base from the DNA duplex (6, 9–11). In the case of the UDGs, there is no apparent facility for a gross conformational change in the enzyme, and the only way that uracil can enter into the specific pocket is for the base to become extrahelical. This has recently been shown structurally for UDG (6).

The phenomenon of "base-flipping" has yet to be experimentally deconvoluted from the reaction as a whole, and it is still open to debate as to whether the extrusion of DNA bases from a duplex is spontaneous or is actively promoted by the DNA modification or repair enzymes (12). Structural analysis alone is unable to distinguish the two possible mechanisms. Mutation of active site residues to yield catalytically inactive mutant proteins coupled with careful binding studies may well yield a satisfactory answer to this question; it is with this question in mind that these studies were undertaken. Both of the residues implicated in catalysis by structural studies, aspartic acid-88 and histidine-210, were mutated to asparagine and used in binding studies to a range of oligonucleotides using surface plasmon resonance. This system is a powerful means of attempting to deconvolute the mode of substrate recognition and binding by UDG in the absence of catalysis.

EXPERIMENTAL PROCEDURES

Construction of Recombinant Mutants of HSV1 UDG—Aspartic acid-88 and histidine-210 of the recombinant HSV1 UDG were both mutated to asparagine (D88N and H210N, respectively) to abolish catalytic activity. Oligonucleotide 18-mers were designed to change the codons in the open reading frame from aspartic acid and histidine,

* This work was supported by the Cancer Research Campaign. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed. Tel.: 44-171-380-7372; Fax: 44-171-380-7193; E-mail: pearl@bsm.biochemistry.ucl.ac.uk.

** Present address: Dept. of Crystallography, Birkbeck College, Malet St., London, WC1E 7HX UK.

[1] The abbreviations used are: UDG, uracil-DNA glycosylase; HSV1, herpes simplex virus type 1; ss, single-stranded; ds, double-stranded; Ugi, uracil-DNA glycosylase inhibitor; RU, resonance units; E+DNA(U), enzyme in the presence of uracil-DNA; E:DNA(U), enzyme in complex with uracil-DNA; E:U(DNA), enzyme in complex with a DNA-uracil.

respectively, to asparagine with the substitution of just one nucleotide in each case. The oligonucleotides were designed such that the mismatch was located in the middle of the sequence. The method used for mutagenesis was the Altered Sites II system (Promega) and was followed as per protocol. The mutagenesis was effected by subcloning the HSV1 UDG open reading frame from the original expression vector pTS106· (13) using *Nco*I and filling the overhang with Klenow purification of DNA by Geneclean (Anachem) and final excision of the open reading frame using *Hin*dIII, and inserting it into the mutagenesis vector supplied with the kit (pALTER1) into *Sma*I and *Hin*dIII, thus recreating the *Nco*I site for subsequent recloning into pTrc99A following mutagenesis. Unique restriction sites were introduced by mutagenesis (*Eco*O109I for D88N, and *Bst*BI for H210N), and these were used to screen for positive mutant DNAs. Positively identified mutants were cloned as *Nco*I to *Hin*dIII fragments in pTrc99A (Pharmacia Biotech Inc.) cut with the same enzymes.

Expression and Purification of Mutant Proteins—Expression was performed in the *Escherichia coli* strain BL21(DE3), and standard assays were carried out for UDG activity (14). Inactive mutant clones were stored as 50% glycerol stocks at −80 °C. Expression of D88N mutant resulted in low level expression of insoluble protein under the standard expression conditions (13), and optimum expression was obtained by growing the bacteria to $A_{600\ nm}$ = 0.8–1.0 at 28 °C, before inducing with 0.5 mM isopropyl-β-D thiogalactopyranoside for 36 h. The H210N mutant was expressed under the standard conditions (13). Purification was by the following method. Following lysis of cells and partition of soluble and insoluble fractions, (13), the clarified supernatant was applied to a two-column system (60 ml Q-Sepharose outlet into the inlet of 60 ml SP-Sepharose) equilibrated with 20 mM Tris-HCl, 10 mM EDTA, pH 8.3, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol chilled in an ice-bath. A wash of 800 ml of the equilibration buffer was performed subsequent to loading, and a further 400 ml wash using the same buffer was then performed on the SP-Sepharose column alone. A 0 to 1.6 M sodium chloride gradient was run through the SP-sepaharose column over 7 column volumes. Fractions containing the bulk of mutant UDG, as judged by SDS-polyacrylamide gel electrophoresis analysis, were pooled and diluted such that the final sodium chloride concentration was less than 50 mM, using buffer B (20 mM Tris-HCl/10 mM EDTA pH 8.3, 10% glycerol, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol). The diluted protein was loaded onto a 25-ml poly-U-Sepharose column, pre-equilibrated in buffer B. A wash of 5-column volumes of buffer B with 100 mM sodium chloride was performed after loading, and then a gradient of 100–250 mM sodium chloride over 5-column volumes was performed. UDG mutant protein was eluted in a 2 M sodium chloride step. Dialysis was performed twice against 100 volumes of 10 mM Tris-HCl, pH 8.3, 0.1 mM phenylmethanesulfonyl fluoride. The proteins were shown to be greater than 95% pure after this procedure, with D88N having a final yield of 2 mg/liter bacterial culture and with H210N having a final yield of 15 mg/liter bacterial culture.

Crystallization of Mutant UDG Proteins—Proteins were concentrated following purification in Amicon-stirred ultrafiltration cells using PM10 membranes (Amicon Corp.). The D88N mutant was far less soluble than H210N, and was used for crystallization trials at 15 mg/ml. The H210N mutant was used at 35 mg/ml. Purified concentrated proteins were 0.22-mm filtered in Ultrafree MC microcentrifuge cups (Millipore), and phenylmethanesulfonyl fluoride and sodium azide were added to final concentrations of 0.1 mM and 0.02% (w/v), respectively. The protein stocks were then stored at 4 °C where they were stable for several months. Both mutants were put through crystallization trials using the Hampton research crystal screen, implemented as 1:1 microbatch mixtures under paraffin oil in Terazaki plates (15). Plates were stored at 16 °C in the dark. Similar screens were also performed for proteins mixed at ratios of 1:2 (protein:ligand) with nucleotides and oligonucleotides.

Preparation of Immobilized Ugi Protein—The Ugi protein was prepared as described previously (16), and immobilized on Affi-Gel 15 cationic beads (Bio-Rad) using the following protocol. Beads, stored as supplied at −20 °C, were allowed to reach room temperature over 30 min with occasional inversion of the bottle. After thoroughly resuspending the beads by inversion and agitation, a volume of approximately 120 μl was withdrawn with a 1-ml micropipettor using a wide bore tip (made by sawing off one-third of the tapered end) and transferred to a 1.5 ml microcentrifuge tube. The tube was pulsed for 30 s at 16,000 × *g* to pellet the beads, and the bulk of the isopropanol-rich medium was discarded. A volume of 500 μl of ice-cold deionized water was added to the beads, and the tube was vortexed for 5 s. The tube was pulsed as described, and the water was discarded. This was repeated twice more, and then finally with 50 mM HEPES-NaOH, pH 7.0. Upon discarding the HEPES-NaOH, 400 μl of HEPES-NaOH, pH 7.0, containing 3 mg of purified Ugi protein was added to the beads. The tube was agitated gently to suspend the beads in the protein solution, and the agitation was continued on a shaker for 90 min at room temperature. The protein binds to the beads through lysine ϵ-amino groups during this step. The tube was pulsed as described previously, and the supernatant was removed and stored. The beads were washed three times with 500 μl of 50 mM HEPES-NaOH, pH 7.0, as described earlier, with the supernatant being stored after each spin. After removing the supernatant following the final spin, 10 μl of freshly prepared ethanolamine, pH 8.0, were added and mixed in by tapping the tube gently; the tube was allowed to stand for an hour at room temperature. This step blocks any beads that have not bound protein. The tube was pulsed as described previously, discarding the supernatant, and was washed twice with 400 μl of 50 mM HEPES-NaOH, pH 7.0, discarding the supernatant each time. The beads were then ready to use.

Synthesis and Purification of Oligonucleotides Used in These Binding Studies—The oligonucleotide sequence used in these experiments was 5′-biotin-CCGAATCAGTTCACTTCNAGCCGAGGTATTTAGCC, for oligonucleotides ssC and ssU, where N is C in ssC and N is U in ssU. The nonbiotinylated oligonucleotides were 5′-GGCTAAATACCTCGGCTNGAAGTGAACTGATTCGG where N is either A or G to form the duplexes dsU/A and dsU/G, respectively, with the biotinylated strand ssU, and the duplex dsC/G with ssC. Oligonucleotide synthesis was performed on an Applied Biosystems 394 DNA synthesizer on the 0.2-μm scale using cyanoethyl phosphoramidite chemistry. Standard DNA synthesis reagents and cyanoethyl phosphoramidite monomers were obtained from Applied Biosystems, Ltd. The biotin phosphoramidite was obtained from Cruachem Ltd. and used as a 0.15 M solution in anhydrous acetonitrile; the coupling time was extended to 3 min. Stepwise coupling efficiencies were measured automatically on the synthesizer by trityl analysis, and all monomers were coupled at greater than 98%. Oligonucleotides were deprotected in concentrated aqueous ammonia for 8 h at 55 °C. High performance liquid chromatography purification was carried out on a Gilson model 306 high performance liquid chromatography system using a Brownlee Aquapore octyl reverse phase column (10 × 250 mm) with a flow rate of 3 ml/min and a gradient of 0–75% buffer B over a period of 30 min. (Buffer A, 0.1 M triethylammonium acetate; buffer B, 0.1 M triethylammonium acetate with 25% acetonitrile). In the case of oligonucleotides labeled with biotin, the correct product was the major peak (final peak to elute from the column). After high pressure liquid chromatography purification, the major product was evaporated to dryness and desalted using a Pharmacia NAP 10 column (Sephadex G25) according to the manufacturer's instructions.

Experimental Assay Procedure Using Surface Plasmon Resonance—The principle of operation of the BIAcore biosensor and its use in analyzing protein-DNA interactions have been described before (17, 21). All interactions were analyzed in binding buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20) at a constant flow rate of 5 μl/min and at a constant temperature of 25 °C. Biotinylated oligonucleotides were injected over a streptavidin-coated sensor chip (SA5, Pharmacia Biosensor) until a suitable level was achieved (see "Results"). For the formation of double-stranded oligonucleotides, nonbiotinylated DNA was injected until no more increase in binding to the immobilized single-stranded DNA could be observed. Bound protein was eluted from the DNA by a short pulse (5 μl) of 0.05% SDS. This regeneration procedure did not alter to any measurable extent the ability of the immobilized DNA to bind protein in subsequent cycles.

Analysis of the data was performed using the evaluation software supplied with the instrument. To eliminate small "bulk" refractive change differences at the beginning and end of each injection (due to small differences in buffer composition of the stock protein solutions), a control sensorgram obtained over a nonbinding surface was subtracted for each protein injection. For obtaining the association and dissociation rate constants the following equations were used, respectively.

$$R = R_{eq}[1 - e^{-(k_a C + k_d)(t - t_0)}] \quad \text{(Eq. 1)}$$

and

$$R = R_0 e^{-k_d(t - t_0)} \quad \text{(Eq. 2)}$$

R, R_0, and R_{eq} are the response at time t, t_0, and at equilibrium, respectively; C, the concentration of protein; k_a, the association rate constant; k_d, the dissociation rate constant; and t_0, the start time of the dissociation or association. For the determination of the equilibrium dissociation constant (K_D) from binding experiments

$$R = \frac{R_{max} \cdot C}{K_D + C} \qquad \text{(Eq. 3)}$$

was used (where R_{max} is the maximum response level).

RESULTS

Confirmation of Tertiary Structure of Mutant Proteins—The integrity of the tertiary structures of the mutant proteins was verified in the absence of catalytic activity against a standard substrate routinely used to assay UDGs (14) by the following methods. First, *Bacillus subtilis* bacteriophage PBS1 Ugi protein (16) immobilized on Affi-Gel 15 beads, was shown by SDS-polyacrylamide gel electrophoresis to sequester and by standard assay (14) to inactivate wild-type HSV1 UDG. Similarly immobilized Ugi protein was shown by SDS-polyacrylamide gel electrophoresis to sequester both mutants with similar affinity to its capture of wild-type enzyme. To remove the UDG or mutants from the Ugi protein, it was necessary to heat the beads in the presence of SDS. As a control, whole protein from an *E. coli* whole cell lysate was shown by SDS-polyacrylamide gel electrophoresis to bind very weakly apart from *E. coli* UDG, which was retained strongly. This strongly suggests that the DNA-binding channel and overall fold of the mutant proteins do not significantly differ from that of the wild-type enzyme.

The second method used to indicate correct folding was using crystallization and x-ray diffraction. Both D88N and H210N mutants were crystallized using the Hampton research crystal screen following purification, and the latter also formed rectangular rod-like crystals spontaneously *in situ* after 10 days at 4 °C, average size 80 mm thick by 200 mm long, which diffracted to 2.5 Å on a laboratory source. The spacegroup was found to be $P2_1$ with unit cell a = 42.9 Å, b = 61.6 Å, c = 43.9 Å, and β = 93.05, with a solvent content of 58% and 1 molecule in the asymmetric unit. Due to the fact that this is a monoclinic crystal form, a complete dataset has not yet been collected, and a structure is not yet available. The needle-like crystals of the D88N and H210N mutants obtained in crystal screens from polyethylene glycol 4000/buffer mixtures were too small for x-ray analysis, but co-crystals of the D88N mutant with the single-stranded trideoxynucleotide pdTpdTpdU, resulted in six-sided plates with average dimensions of 100-mm across by 20–50-mm thick. These crystals are seen to diffract beyond 2.6 Å at a synchrotron radiation source. Further characterization is in progress. The D88N mutant also yields rod-like crystals with the nucleotides 3′-dUMP and 5′-dUMP, and both mutants yield large orthorhombic rods with the trideoxynucleotide pdTpdTpdT identical to those obtained with the wild-type enzyme. Crystals of macromolecules, which diffract to medium and high resolution, are a good indication that there is inherent order and homogeneity in the components of the lattice. Morphological changes of protein crystals in the presence of potential ligands is a good indication that productive binding has taken place, demonstrating that the protein is correctly folded.

Binding of Proteins to Immobilized Oligonucleotides—The BIAcore biosensor was used to measure the specificity and affinity of the interactions between mutant or wild-type enzyme and single- or double-stranded oligonucleotides. In this system, the biotinylated oligonucleotide is immobilized on a streptavidin-coated dextran layer attached to a sensorchip, and the protein is injected at a constant flow rate. The biosensor measures refractive index changes close to the dextran layer, which in turn correspond to changes in the amount of protein bound to the surface. The plot of bound protein (measured in arbitrary resonance units (RU)) *versus* time is called a sensorgram and can be used to derive kinetic or equilibrium constants. This method has been used before to study other protein-DNA interactions (17–19). To immobilize double-stranded oligonucleotides, one of the strands was biotinylated and attached to the surface first and then the other strand was injected at saturating amounts until no further binding was observed (see "Experimental Procedures"). Complete formation of double-stranded DNA was verified by observing approximate doubling of the static resonance signal after the annealing of the second strand (Fig. 2). There was no measurable dissociation of DNA from the surface even after many cycles of binding and regeneration. Measurements of protein-DNA interactions were obtained by passing protein solutions over the immobilized oligonucleotides on the surface of the chip and analyzing the resulting sensorgrams.

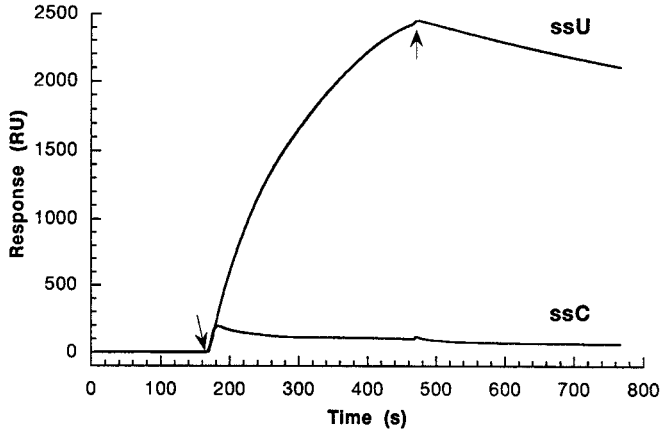

FIG. 1. **Specificity of binding.** Interaction of the D88N mutant with a streptavidin-coated sensorchip saturated with biotinylated ssC and ssU oligonucleotides. The *arrows* point to the start and end of the injection.

Specificity of Binding—Initial experiments were designed to check the specificity of binding to uracil-containing DNA and therefore the maximum possible amounts of oligonucleotides ssC and ssU (see "Experimental Procedures") were immobilized on the streptavidin-coated chip (approximately 1100 RU). The mutant D88N and H210N proteins were then passed over both surfaces. As can be seen in Fig. 1 even with these high amounts of immobilized DNA, there was no significant binding to oligonucleotide ssC, demonstrating the absolute requirement for uracil in specific recognition. When the wild-type enzyme was tested, no binding was observed to oligonucleotides ssC or ssU. The most obvious interpretation for this observation would be the rapid removal of uracil by the active enzyme making it impossible to detect a stable enzyme·DNA complex. This was confirmed by testing the binding of mutant enzyme before and after injection of the wild-type enzyme. Fig. 3 shows that following application of the active enzyme, the mutant D88N was no longer capable of interacting with the surface, indicating that almost all of the available uracil had been removed. The same result was obtained with mutant H210N (not shown). The much lower binding displayed after removal of the uracil by the wild-type enzyme indicates that neither the wild-type nor the mutants have significant affinity for the abasic sites generated in the single-stranded DNA by the uracil-excision reaction.

Comparison between Single- and Double-stranded Oligonucleotides—Two different nonbiotinylated oligonucleotides (ssG and ssA, see "Experimental Procedures") were used to create double-stranded DNA (dsU/G and dsU/A) using immobilized, biotinylated oligonucleotides ssU, and dsC/G using immobilized, biotinylated ssC. As with ssC, dsC/G showed no indications of binding with the wild-type enzyme nor either of the mutants (results not shown). Fig. 4 shows the relative binding

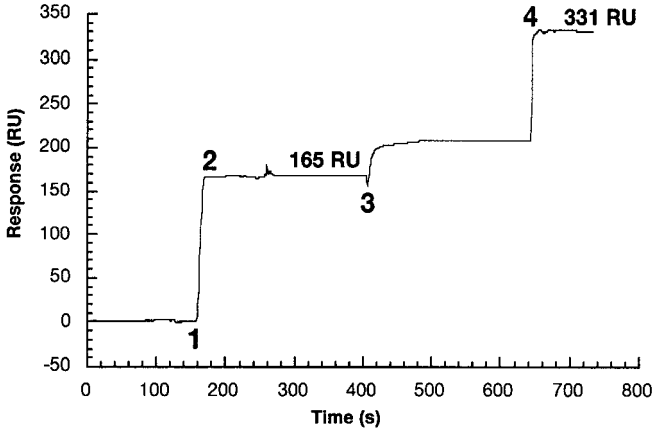

FIG. 2. **Generation of duplex DNA *in situ*.** The RU values represent the magnitude of the response compared with the baseline before the first injection. An exact doubling of the response was observed after injection of the second oligonucleotide. Additional injections did not increase the response any further. *Marked points* indicate the following: *1*) beginning of injection of biotinylated single-strand oligonucleotides; *2*) end of injection; *3*) beginning of injection of second single-strand oligonucleotides; and *4*) end of injection (a high concentration of oligonucleotides was used, and because the stock was dissolved in water, dilution of the buffer occurred resulting in the sudden "jump" of the response at *point 4*. This is not so obvious at *point 3* because of the fast binding of the oligonucleotides).

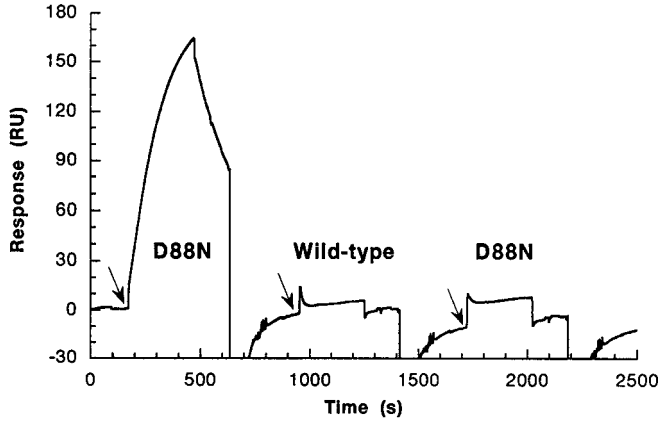

FIG. 3. **Uracil excision by the wild-type enzyme abolishes UDG binding to ssDNA.** Immobilized ssU oligonucleotide was allowed to interact with D88N before and after injection of an equal concentration of wild-type enzyme. The *arrows* point to the start and end of each injection. The sharp decreases in the signal after the interaction are due to the regeneration step to remove bound protein (see "Experimental Procedures").

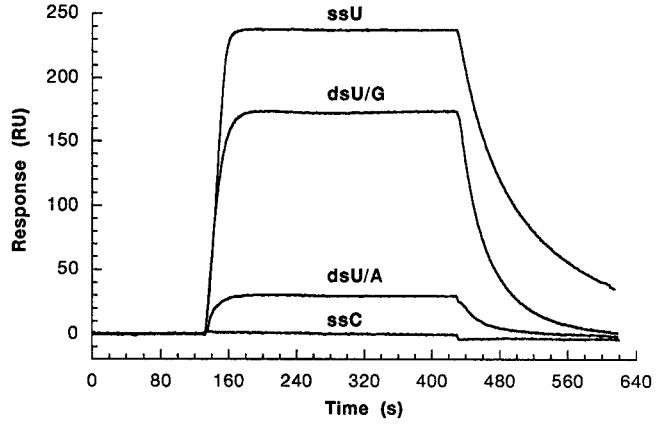

FIG. 4. **Comparison of binding to ssDNA and dsDNA.** Binding of H210N to four surfaces coated with the same quantity of the indicated oligonucleotides. Similar results were obtained for the D88N mutant.

of a single concentration of mutant H210N to four surfaces coated with the same amount (approximately 200 RU of single-strand oligonucleotides) of ssC, ssU, dsU/G and dsU/A (similar results were obtained with both mutants, data not shown). Both double-strand oligonucleotides bound protein with reduced affinity compared with ssU, and binding to dsU/A was considerably reduced compared with dsU/A. As with the single-stranded uracil-containing oligonucleotides, treatment with wild-type UDG effectively abolished subsequent binding by wild-type or mutant enzymes, indicating that neither the wild-type nor mutant enzymes display significant affinity for the abasic sites in double-stranded DNA produced by the uracil-excision reaction (Fig. 5).

To obtain a quantitative comparison, we took advantage of the fact that equilibrium was reached for all these interactions, and therefore the equilibrium dissociation constant could be determined by injecting a range of concentrations and plotting the response at equilibrium *versus* the concentration of injected protein. For these assays as well as for kinetic analysis (see below), the level of immobilized oligonucleotides was further reduced to approximately 40 RU to avoid possible artifacts due to mass transport limitations. Furthermore, it has been suggested (20) that the measurement of K_D values by equilibrium binding assays is affected by the amount of "receptor" used, and considerable deviations can be observed from the true values if the levels are too high. Whereas the overall response was small, the data obtained in this way fitted very well to an equation describing a simple bi-molecular interaction obeying the law of mass action (see "Experimental Procedures"). The results of these experiments for the mutant H210N are summarized in Table I. An example of the fits obtained using Equation 3 is shown in Fig. 6 for binding to the ssU oligonucleotides. This figure also demonstrates that there is no substantial difference between the two mutant enzymes, D88N and H210N, and similar good fits were obtained for the other oligonucleotides (Table I).

Estimation of Kinetic Constants—To analyze the interaction with the uracil-containing oligonucleotides further, the kinetic constants were determined. An example is shown in Fig. 7 for the binding of the H210N mutant to the dsU/G oligonucleotides. Fig. 7 shows the fit of the association rate (*A*) and that of the dissociation rate (*B*) obtained using Equations 1 and 2 (see "Experimental Procedures"), which describe a homogeneous single-site interaction. Data were obtained with several different concentrations of protein and for the three different oligonucleotides and are summarized in Table I. In all cases the fits were statistically highly significant, and there was no evidence of deviations due to mass transport effects, although these were observed when higher amounts of oligonucleotides were immobilized (not shown). The interaction with the ssU oligonucleotides is characterized by the fastest association rate and the slowest dissociation rate. The difference in affinity between the two double-strand oligonucleotides is mainly due to an over 10-fold faster association rate for dsU/G compared with dsU/A. From the kinetic constants, the equilibrium dissociation constant could also be independently estimated from the equation $K_D = k_d/k_a$. As can be seen in Table I there is very good agreement between the K_D values calculated in this way and those determined directly by the equilibrium binding experiments described above, confirming the validity of the fits.

DISCUSSION

Structural studies show unambiguously that recognition of uracil by UDGs requires insertion of the uracil base into a pocket in the enzyme, which for dsDNA can only be achieved by

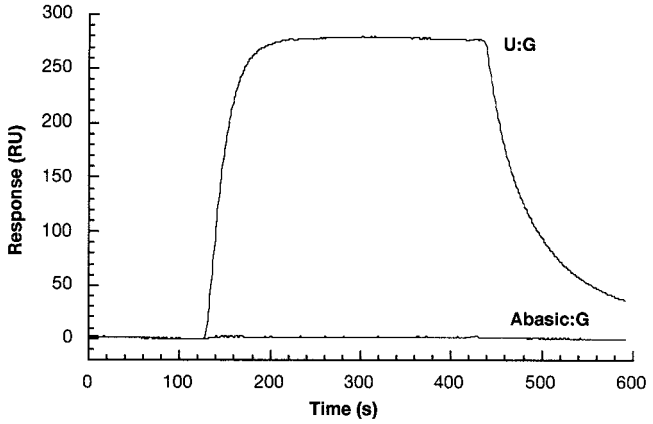

FIG. 5. **Uracil excision abolishes UDG binding to dsDNA.** Binding of D88N UDG to immobilized dsU/G oligonucleotide before and after uracil excision by wild-type UDG. In contrast with the high affinity for the uracil-containing duplex, neither wild-type nor mutant UDGs are retained on the product oligonucleotide containing an abasic site.

TABLE I

Kinetic and equilibrium dissociation constants of the interaction between the H210N mutant and oligonucleotides

The estimates of the kinetic constants are shown as average ± standard deviation (S.D.) of n independent determinations done at different concentrations of H210N protein. The calculated equilibrium dissociation constant, K_D, was obtained from the equation $K_D = k_d/k_a$ for each experiment and then averaged. The experimental determination of K_D was obtained using Equation 3, and the standard error (S.E.) for the fit is indicated.

Oligonucleotide	n	k_a ± S.D.	k_d ± S.D.	Calculated K_D ± S.D.	Experimental K_D ± S.E.
		$M^{-1}\ s^{-1}$	s^{-1}	nM	
ssU	16	4.03×10^6	0.0175	4.65	6.01
		$\pm 1.22 \times 10^6$	±0.0029	±1.18	±0.13
dsU/G	10	5.92×10^5	0.0336	57.4	56.5
		$\pm 0.74 \times 10^5$	±0.0025	±7.2	±2.0
dsU/A	10	3.82×10^4	0.0553	1493	1916
		$\pm 0.67 \times 10^4$	±0.0044	±325	±81

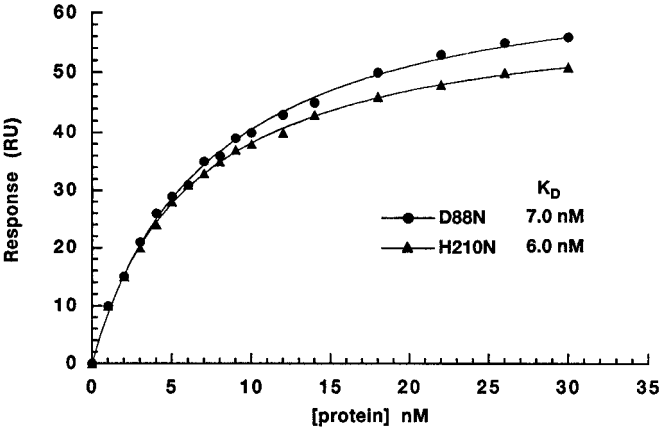

FIG. 6. **Comparison of the binding of the two mutant enzymes to ssU.** A range of protein concentrations was injected, and the response at equilibrium was plotted *versus* the concentration. The data were analyzed using Equation 3 (see "Experimental Procedures") to obtain estimates of the equilibrium dissociation constant, K_D.

"flipping" the deoxyuridine nucleotide into an extrahelical conformation (4–6). However, these structural results cannot explain the mechanism of this recognition process. Two questions in particular need to be answered. First, does the enzyme locate uracils by scanning the DNA in a "one-dimensional" diffusion process (22) or by simple bimolecular collision; and second, does the enzyme actively promote the flipping of the uracil from a

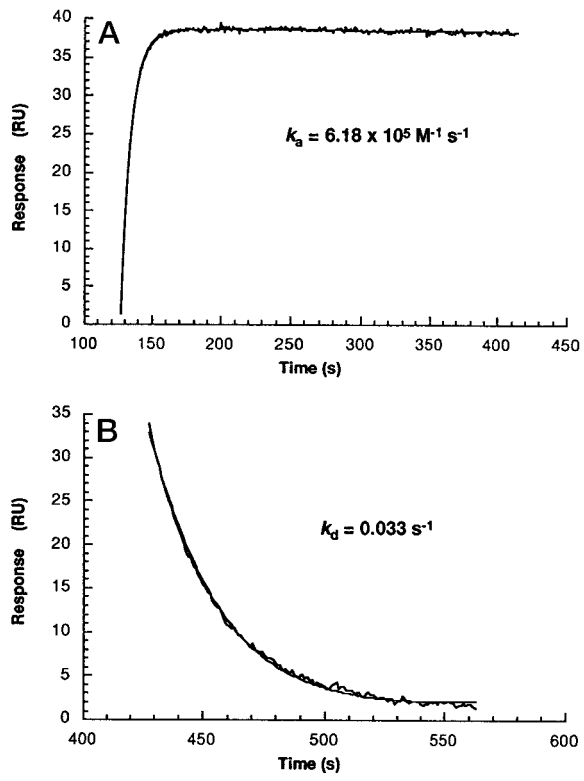

FIG. 7. **Determination of rate constants.** The "association" (*A*) and "dissociation" (*B*) phases of a sensorgram obtained by interaction of 250 nM H210N with immobilized dsU/G were analyzed using Equations 1 and 2, respectively (see "Experimental Procedures"). The fits are shown superimposed on the raw data.

stacked conformation, or does it recognize spontaneously flipped bases?

The affinity of native and mutant UDG, for single-stranded or double-stranded uracil-free DNA, is too low to be measured using the BIAcore system. This indicates that the residence time of the enzyme on ordinary DNA is very short and makes it unlikely that the enzyme is able to scan along the DNA for any appreciable length. That the rates of association and dissociation from uracil-DNA are very well modeled by single processes (see Equations 1 and 2 and Fig. 7) rather than by the biphasic model required if scanning were in operation (*i.e.* E + DNA(U) → E:DNA(U) → E:U(DNA)) further suggests that direct binding of uracil in the DNA is the only process taking place.

Whereas our observations appear to rule out facilitated diffusion as an option for locating uracil in DNA, substrate location might be made more efficient by electrostatic steering of the positively charged DNA-binding face of UDG (4, 6) toward the negatively charged DNA molecule during bimolecular collision events. Such an oriented "hopping" process (22, 23) would certainly serve to reduce the effective dimensionality of the search in a similar, albeit more crude, manner than that achieved by one-dimensional diffusion. This would also make sense of data suggesting that UDG interacts with DNA in a "distributive" manner (24), but shows effects that would tally with a scan length of 1.5–2 kilobases prior to dissociation from DNA (25). A hopping mechanism is also supported by the structural data (6), which suggests that UDG would have to dissociate from DNA after a uracil-excision event to allow the free uracil product to be released, a requirement that would be difficult to reconcile with a sliding mechanism.

Introduction of a single uracil base into the single- and double-stranded oligonucleotides produced a dramatic increase

in the affinity of the catalytically inactive mutants for the single- and double-stranded DNA over that observed for uracil-free DNA. The relative affinity for uracil-DNA was strongly dependent on the structural context of the uracil, in the order ssU-DNA > dsU/G-DNA ≫ dsU/A-DNA, for both mutants. As neither the native nor the mutant enzymes showed any significant affinity for the abasic site that results from the uracil-excision reaction (Figs. 2 and 5), this increased affinity can be attributed to the interactions between the enzyme and the destacked uracil base, which are essentially identical in all three systems. This order of relative affinity can be understood in terms of the work required to destack the uracil and make it available to the binding pocket; being least in ssDNA where no base pair is disrupted, more for the "wobble" G:U base pair, and most for the fully stacked Watson-Crick A:U base pair. Affinity for uracil in DNA rather than for any features of DNA *per se* is consistent with the ability of UDG to act on ssDNA as well as dsDNA, more or less irrespective of sequence context. The differences in affinity for the DNA molecules between the two mutants are very small, confirming the suggestion that both of these residues have purely catalytic roles in the enzyme mechanism (4, 6).

Neither the native nor the mutant enzymes showed any apparent affinity for abasic sites, and binding of the active wild-type enzyme to uracil-DNA ligands was not detectable. Association with uracil-DNA and its subsequent hydrolysis by the wild-type enzyme, though undetected, must have taken place as demonstrated by the abolition of subsequent binding of the catalytically inactive mutants. The lack of a detectable enzyme·DNA complex during catalysis is consistent with the observed fast on-rate and low affinity for the abasic reaction product and suggests that the actual rate of bond hydrolysis is not limiting.

Two broad classes of base-flipping enzyme can be defined; sequence specific DNA-modifying enzymes, and sequence-independent DNA-modifying enzymes. A sequence-specific enzyme, such as a restriction methyltransferase, interacts with an extrahelical base within a defined sequence context that can be recognized in a fully stacked conformation. Once bound to its cognate DNA sequence, a sequence-specific enzyme can simply wait for the thermal breathing motion of the DNA to flip its target base spontaneously (9, 12). In contrast, a sequence-independent enzyme, such as uracil-DNA glycosylase, must recognize the presence of its target base directly. Unlike some lesions recognized by DNA repair enzymes, which cause significant distortion in the structure of the double helix, uracil is a potentially difficult target to detect. In a G:U mismatch, the wobble of the uracil into the major groove might enable recognition *in situ* of the presence of uracil, however, a U:A base pair, which is also efficiently repaired, is a fully stacked Watson-Crick base pair with no protrusions. The pattern of functional groups presented by an A:U base pair in the major groove is different from the patterns presented by either of the normal G:C or A:T base pairs and might therefore provide a means for *in situ* recognition. However, the major groove pattern for A:U is also very different from that presented by G:U, and it is difficult to imagine how an enzyme would achieve simultaneous specific major (or minor) groove recognition for these very different base pairs in dsDNA while also recognizing unpaired uracil in ssDNA. A much simpler model, consistent with the relative strengths of binding we observe for uracil in these different contexts, would suggest that UDG is recognizing "flipped out" uracil. These findings are supported by the rates for association and dissociation of either mutant from the different oligonucleotides. The association rates are markedly different in the order ssU-DNA > dsU/G-DNA ≫ dsU/A-DNA suggesting that the enzyme·U-DNA complex formation is dependent on the relative strength of any base pairing. The dissociation rates are relatively similar and suggest that once the complex is formed, the enzyme dissociation is from an essentially identical complex in all cases.

The limited interaction between UDG and DNA-containing bases other than uracil is a function of the exquisitely selective specificity pocket of the enzyme, which will only allow uracil and some close analogues to enter and bind (4–6). The displacement of several bound water molecules from the pocket upon binding of uracil is entropically favorable, and the formation of three hydrogen bond contacts to uracil in the active site (4, 6), as well as the insertion of a hydrophobic side-chain into the space left in the duplex by the flipped nucleotide (6), is a stable arrangement. Further stabilization will be provided by the DNA-binding channel, which makes favorable contacts with the distorted backbone geometry generated by a flipped nucleotide (6). This suggests that molecular impact of the enzyme onto the DNA and thermal motion of DNA, together with the stabilization of the helix distortion provided by the DNA-binding channel of the enzyme, would be sufficient to drive the complete process of recognition, binding, and catalysis without penalty.

The question of whether UDGs are indeed facilitating inherent base flipping in the duplex, or just binding to opportunistically flipped-out bases cannot be demonstrated solely from our results and the available structural information. However, this could be addressed by measuring energy changes on interaction using a technique such as microcalorimetry. This approach is currently being investigated for the mutant proteins used in this study.

REFERENCES

1. Mosbaugh, D. W. (1988) *Rev. Biochem. Tox.* **9,** 69–130
2. Lindahl, T. (1993) *Nature* **362,** 709–715
3. Focher, F., Verri, A., Verzeletti, S., Mazzarello, P., and Spadari, S. (1992) *Chromosoma (Berl.)* **102,** S67–S71
4. Savva, R., McAuley-Hecht, K., Brown, T., and Pearl, L. (1995) *Nature* **373,** 487–493
5. Mol, C. D., Arvai, A. S., Slupphaug, G., Kavli, B., Alseth, I., Krokan, H. E., and Tainer, J. A. (1995) *Cell* **80,** 869–878
6. Slupphaug, G., Mol, C. D., Kavli, B., Arvai, A. S., Krokan, H. E., and Tainer, J. A. (1996) *Nature* **384,** 87–92
7. Savva, R., and Pearl, L. H. (1995) *Nat. Struct. Biol.* **2,** 752–757
8. Mol, C. D., Arvai, A. S., Sanderson, R. J., Slupphaug, G., Kavli, B., Krokan, H. E., Mosbaugh, D. W., and Tainer, J. A. (1995) *Cell* **82,** 701–708
9. Klimasauskas, S., Kumar, S., Roberts, R. J., and Cheng, X. D. (1994) *Cell* **76,** 357–369
10. Vassylyev, D. G., Kashiwagi, T., Mikami, Y., Ariyoshi, M., Iwai, S., Ohtsuka, E., and Morikawa, K. (1995) *Cell* **83,** 773–782
11. Park, H. W., Kim, S. T., Sancar, A., and Deisenhofer, J. (1995) *Science* **268,** 1866–1872
12. Roberts, R. J. (1995) *Cell* **82,** 9–12
13. Savva, R., and Pearl, L. H. (1993) *J. Mol. Biol.* **234,** 910–912
14. Koulis, A., Cowan, D. A., Pearl, L. H., and Savva, R. (1996) *FEMS Microbiol. Lett.* **143,** 267–271
15. Chayen, N. E., Shaw-Stewart, P. D., Maeder, D. L., and Blow, D. M. (1990) *J. Appl. Crystallogr.* **23,** 297–302
16. Savva, R., and Pearl, L. H. (1995) *Proteins Struct. Funct. Genet.* **22,** 287–289
17. Spanopoulou, E., Zaitseva, F., Wang, F.-H., Santagata, S., Baltimore, D., and Panayotou, G. (1996) *Cell* **87,** 263–276
18. Fisher, R. J., Fivash, M., Casas Finet, J., Erickson, J. W., Kondoh, A., Bladen, S. V., Fisher, C., Watson, D. K., and Papas, T. (1994) *Protein Sci.* **3,** 257–266
19. Bondeson, K., Frostell Karlsson, A., Fagerstam, L., and Magnusson, G. (1993) *Anal. Biochem.* **214,** 245–251
20. Chang, K.-J., Jacobs, S., and Cuatrecasas, P. (1975) *Biochim. Biophys. Acta* **406,** 294–303
21. Jönsson, U., Fägerstam, L., Roos, H., Rönnberg, J., Sjölander, S., Stenberg, E., Stahlberg, R., Urbaniczky, C., Östlin, H., and Malmqvist, M. (1991) *BioTechniques* **11,** 520–527
22. von Hippel, P. H., and Berg, O. H. (1989) *J. Biol. Chem.* **264,** 675–678
23. Berg, O. G., Winter, R. B., and von Hippel, P. H. (1981) *Biochemistry* **20,** 6926–6948
24. Purmal, A. A., Lampman, G. W., Pourmal, E. I., Melamede, R. J., Wallace, S. S., and Kow, Y. W. (1994) *J. Biol. Chem.* **269,** 22046–22053
25. Higley, M., and Lloyd, R. S. (1993) *Mutat. Res.* **294,** 109–116

Direct Measurement of the Substrate Preference of Uracil-DNA Glycosylase

George Panayotou, Tom Brown, Tom Barlow, Laurence H. Pearl and Renos Savva

J. Biol. Chem. 1998, 273:45-50.
doi: 10.1074/jbc.273.1.45