Site-directed Mutagenesis and Characterization of Uracil-DNA Glycosylase Inhibitor Protein

ROLE OF SPECIFIC CARBOXYLIC AMINO ACIDS IN COMPLEX FORMATION WITH ESCHERICHIA COLI URACIL-DNA GLYCOSYLASE

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Bacteriophage PBS2 uracil-DNA glycosylase inhibitor (Ugi) protein inactivates uracil-DNA glycosylase (Ung) by acting as a DNA mimic to bind Ung in an irreversible complex. Seven mutant Ugi proteins (E20I, E27A, E28L, E30L, E31L, D61G, and E78V) were created to assess the mechanism involved in Ung association and irreversible complex formation. The secondary and tertiary structures of free Ugi were recently determined by solution state multi-dimensional nuclear magnetic resonance and compared with the unbound Ugi structure. Structural and functional analysis of these proteins have elucidated the two-step mechanism involved in Ung-Ugi association and irreversible complex formation.

The Bacillus subtilis bacteriophage PBS1 and -2 exhibit a unique genetic system that naturally contains uracil in place of thymine in a double-stranded DNA genome (1, 2). Stable incorporation of uracil residues into the phage DNA is achieved by the substitution of dUTP for dTTP as precurser in DNA synthesis and the concomitant inactivation of the host uracil-mediated base excision DNA repair pathway (2–4). To block uracil-DNA repair and protect the uracil-containing phase DNA from degradation, an early phage gene (ugi) is expressed that inhibits the B. subtilis uracil-DNA glycosylase. The amino acid sequences of the PBS1 and -2 Ugi proteins appear to be identical (5–7).

The PBS2 ugi gene encodes a small (9,474 dalton), monomeric, heat stable protein of 84 amino acids that inactivates uracil-DNA glycosylases from diverse biological sources (5, 8, 9). The ugi gene product is an unusually acidic protein (12 Glu, 6 Asp) with a pI of 4.2 that migrates anomalously during SDS-polyacylamide gel electrophoresis (5, 10, 11). Ugi inactivates Ung by forming a tightly bound noncovalent complex with 1:1 stoichiometry that is essentially irreversible under physiological conditions (10, 12). Stopped-flow kinetic studies of the Ugi interaction with Escherichia coli Ung indicate that complex formation is accomplished through a two-step binding reaction (12). In the initial step, the association between free Ugi and Ung is characterized by a rapid pre-equilibrium reaction with a dissociation constant (Kd) of 1.3 μm; the second step, the formation of an irreversible complex, is characterized by the rate constant k = 195 s⁻¹. Thus, Ung-Ugi complex formation initiates with a “docking” interaction that facilitates optimal alignment between the two proteins. If correct alignment between Ung and Ugi does not occur, a reversible association will transpire. If, however, proper alignment is achieved, then a “locked” complex quickly follows.

The secondary and tertiary structures of free Ugi were recently determined by solution state multi-dimensional NMR techniques and found to include two α-helices and five anti-parallel β-sheet regions as illustrated in Fig. 1 (13, 14). The five contiguous β-strands are connected by short loop regions to form an anti-parallel β-sheet. Analysis of the electrostatic potential of Ugi revealed several striking features (14). Seven of the 18 acidic amino acid residues (Glu-20, Asp-48, Glu-49, Asp-52, Glu-53, Asp-74, and Glu-78) come together to form a region of high negative potential on one face of the protein. Each of the residues that form this electrostatic region or “knob” are located immediately adjacent to or terminate a β-strand. Two other acidic amino acid residues (Asp-40 and Glu-49) contribute to the region of negative charge.

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The abbreviations used are: ugi and Ugi, bacteriophage PBS1 or 2 uracil-DNA glycosylase inhibitor gene and protein, respectively; Ung, E. coli uracil-DNA glycosylase; NOESY, nuclear Overhauser effect correlation spectroscopy; BSA, bovine serum albumin; HSV-1, herpes simplex virus type-1; bp, base pair(s); kb, kilobase pair(s); TOCSY-HSQC, total correlation spectroscopy-heteronuclear multiple quantum correlation spectroscopy.

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negatively charged residues are located in the loop regions (14). The remaining seven acidic residues include the \( \alpha_1 \)-helix (Ser-9 to Lys-14), \( \alpha_2 \)-helix (Glu-27 to Asp-35), \( \beta_1 \)-strand (Glu-20 to Met-24), \( \beta_2 \)-strand (Ile-41 to Asp-48), \( \beta_3 \)-strand (Glu-53 to Ser-60), \( \beta_4 \)-strand (Ala-69 to Asp-74), and \( \beta_5 \)-strand (Asn-79 to Leu-84).

Asp-61) are also in juxtaposition to the end of \( \beta \)-strands; Glu-78 and Glu-64 reside in the loop regions (14). The remaining seven negatively charged residues are located in the \( \alpha_1 \)-helix (Asp-6, Glu-9, and Glu-11) and \( \alpha_2 \)-helix (Glu-27, Glu-28, Glu-30, and Glu-31). Both the \( \alpha_1 \)- and \( \alpha_2 \)-helix elements project away from the \( \beta \)-sheet and are located on potentially flexible arms of the polypeptide (14). Furthermore, the \( \alpha_2 \)-helix is longitudinally segmented into a hydrophobic face and a negatively charged face where the four glutamic acid residues protrude.

Several lines of evidence suggest that some of the negatively charged amino acid residues of Ugi may act as a DNA mimic and mediate the interaction with Ung. First, UV-catalyzed cross-linking of oligonucleotide \( (dT)_{70} \) to the DNA-binding site of Ung blocked Ugi from forming a Ung-Ugi complex (15). Second, the x-ray crystallographic structure of Ugi in complex with human (16) and HSV-1 (17) uracil-DNA glycosylase reveals that the interfacing surface of Ugi shares shape and electrostatic complementarity to the DNA-binding groove of the enzyme (16, 17). Third, the negative electrostatic knob of Ung exhibits an electrostatic potential of \( \sim 6.6 \) kcal, which is similar to that generated by the negatively charged phosphate backbone of DNA (14). Fourth, the recent x-ray structure of human uracil-DNA glycosylase complexed with a 10-bp oligonucleotide containing a target G-U mispair reveals the DNA complexed at the same site as Ugi (18). Fifth, charge neutralization by carbodiimide-mediated addition of Ugi carboxylic acid residues caused a decrease in inhibitor protein activity (19). Finally, chemical addition of specific glutamic acid residues (Glu-28 and Glu-31) of Ugi located in the \( \alpha_2 \)-helix correlated with the formation of an unstable Ung-Ugi complex (19).

Bennett et al. (12) suggested that after the Ung/Ugi association, the transition to the locked configuration may involve a conformational change in either one or both proteins. Subsequently, Sanderson and Mosbaugh (19) proposed that the locking reaction is caused predominantly by a change in Ugi structure. This position is supported by a comparison of the crystal structures of free human and HSV-1 uracil-DNA glycosylase with the structures of each enzyme in complex with Ugi (16, 17, 20, 21). In both cases, the tertiary structure of the enzyme shows only minor structural changes. In contrast, a comparison of the heteronuclear multiple quantum correlation spectra of free and bound \( [^{15} \text{N}] \)Ugi indicates that many residues of Ugi undergo conformational change upon binding to Ung (14). At present, the tertiary structure of the unbound Ugi protein in solution was determined solely by solution state NMR techniques (14). Also, a comparison of the solution structure of free Ugi with the crystal structure of Ugi complexed with either the human or HSV-1 uracil-DNA glycosylase demonstrates significant structural changes occur in Ugi (14, 16, 17). A more complete understanding of the docking and locking reactions may well be gained by determining the solution state structure of Ugi in complex with Ung.

In the present report we (i) conduct site-directed mutagenesis of seven acidic residues of Ugi; (ii) purify each mutant Ugi protein to apparent homogeneity; (iii) characterize each mutant Ugi with regard to specific activity and Ung-Ugi complex stability and reversibility; (iv) determine the structural similarity between wild type Ugi and the Ugi mutant proteins using NMR methods; (v) compare the free and complexed Ugi solution structures; and (vi) model the interactions in the wild type and mutant Ung-Ugi complexes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases (EcoRI, EcoRV, HindIII, PstI, SacI, and XmnI), T4 polynucleotide kinase, T4 DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs. Isopropyl-1-thio-\( \beta \)-D-galactopyranoside and ScaI were obtained from Life Technologies, Inc. and AcyI came from Promega. \([^{3} \text{H}] \)Leucine and \([^{35} \text{S}] \)methionine were obtained from NEN Life Science Products; \([^{3} \text{H}] \)dUTP was from Amersham Corp., and \([^{13} \text{C}] \)glucose and \([^{15} \text{N}] \)ammonium chloride were from Cambridge Isotope Laboratories.

**E. coli** JM105 was provided by W. Ream (Oregon State University), and **E. coli** Cj236 was obtained from T. A. Kunkel (NIEHS). **Epicurian coli** XL2-Blue ultracompotent cells, phagemid pBluescript II SK(–), and VCS-M13 helper phage were supplied by Stratagene. Plasmid pKK223-3 was obtained from Pharmacia Biotech Inc.; \( pZ 

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**Ugi complexes.**

**Site-directed Mutagenesis of the Uracil-DNA Glycosylase Inhibitor Gene**—The first step in the site-directed mutagenesis procedure involved subcloning of the ugi gene into a pBluescript-based phagemid to produce single-stranded DNA. The pZW7lac EcoRI-HindIII restriction fragment (726 bp) containing the ugi gene (Fig. 2) was inserted into the corresponding EcoRI and HindIII sites of pBluescript II SK(–) using T4 DNA ligase. The resulting phagemid (pAL) was transformed into **E. coli** JM105, plated on LB plates containing 100 \( \mu \)g/ml ampicillin, 40 \( \mu \)g/ml isopropyl-1-thio-\( \beta \)-D-galactopyranoside, and 40 \( \mu \)g/ml 5-bromo-4-chloro-3-indolyl \( \beta \)-D-galactopyranoside, after which pAL DNA was purified from white colonies. **E. coli** Cj236 (dut, ung) was then transformed with phagemid pAL and grown at 37°C in 1.0 liter of 2 \( \times \) YT medium supplemented with 34 \( \mu \)g/ml chloramphenical and 100 \( \mu \)g/ml ampicillin. Upon reaching a cell density of 10^6 cells/ml (1 \( A_{600 \text{ nm}} \) = 8 \( \times \) 10^6 cells/ml), uridine was added to a final concentration of 0.25 \( \mu \)g/ml; VCS-M13 helper phage was added at a multiplicity of infection equal to 1.0, and incubation was continued at 37°C for 1.5 h. Kanamycin (26 \( \mu \)g/ml final concentration) was added to select for infected **E. coli** cells and growth continued for an additional 5.5 h. The culture was centrifuged at 7000 rpm for 15 min at 4°C in a GSA (Sorvall) rotor, and the supernatant fraction was processed to precipitate pAL phage with the addition of 0.25 volume of a 15% PEG-8000 and 2.5 \( \mu \)l NaCl solution. Phage DNA was isolated from the supernatant fraction following extractions with phenol and chloroform/isooamyl alcohol (24:1) and precipitation with ethanol (25). The precipitated DNA was centrifuged at 9500 rpm.
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FIG. 2. Strategy for oligonucleotide-directed mutagenesis of the ugi gene. The EcoRI/HindIII DNA fragment (726 bp) from pZWtac1 contains the ugi structural gene with nucleotide position +1 starting the ATG codon and position +255 terminating the TAA stop codon. Seven oligonucleotides were synthesized as shown that are partially complementary to the antisense sequence of the ugi structural gene over the region depicted. The beginning and ending nucleotide positions are indicated. Noncomplementary bases were engineered at the positions (*) to introduce site-specific mutations that eliminated Glu or Asp residues by amino acid substitution and introduced endonuclease recognition sites into the ugi gene. Endonuclease recognition sites are underlined, and the cleavage sites are indicated (\&). Each oligonucleotide was separately hybridized to uracil-containing pALU(ss) DNA and served to initiate \textit{in vitro} primer extension. Oligonucleotidedirected mutagenesis was conducted as described by Kunkel et al. (23) with modifications indicated under the “Experimental Procedures.” The specific amino acid location and substitution produced by each oligonucleotide is indicated in the \textit{inlaid} table along with the novel restriction endonuclease sites and cleavage location within the 726-bp DNA fragment.

Purification of Uracil-DNA Glycosylase and Inhibitor Protein—Purification of fraction V \textit{[\textit{leucine}-\textit{35S}]}Ugi (13.5 cpm/pmol) was carried out similarly to that described by Bennett et al. (15). Nonradioactive Ugi (fraction V) was purified from \textit{E. coli} CM80/180 cultures (1–1.5 liters) grown in LB medium supplemented with 100 \textit{ug/ml} ampicillin (19). Fraction IV \textit{[\textit{35S}]}methionine-labeled Ugi (40 cpm/pmol) and \textit{[\textit{13C,15N}]}Ugi were purified as described by Sanderson and Mosbaugh (19) from \textit{E. coli} CM80/180 cultures (1–1.5 liters) grown in M9 minimal medium supplemented with 100 \textit{ug/ml} ampicillin, and either 4.2 \textit{mm} of \textit{[\textit{35S}]}methionine or 0.2% [\textit{\textit{13C}}]glucose and 0.17% [\textit{\textit{15N}}]ammonium chloride, respectively. Nonradioactive Ugi and the various site-directed mutants of Ugi were purified following the same procedure, except that bacterial growth occurred in LB medium containing 100 \textit{ug/ml} ampicillin.

Purification of \textit{[\textit{3H}]}Ung-Ugi Complexes—Wild type Ugi or various site-directed mutants of Ugi protein were mixed with \textit{[\textit{3H}]}Ung in buffer A (30 \textit{mm} Tris-HCl (pH 7.4), 1 \textit{mm} EDTA, 1 \textit{mm} dithiothreitol, 5% (w/v) glycerol) containing 50 \textit{mm} NaCl and incubated at 25 °C for 10 min and then at 4 °C for 20 min. Following complex formation, each sample was applied to a DE52 cellulose column equilibrated in buffer A containing 50 \textit{mm} NaCl, washed with equilibration buffer, and step-eluted, as described previously (19). Fractions (1 ml) were collected and samples were analyzed for \textit{3H} radioactivity. The \textit{[\textit{3H}]}Ung-Ugi complex was detected by 18% nondenaturing polyacrylamide gel electrophoresis, and fractions containing complex were pooled and concentrated using a Centricon-10 (Amicon) concentrator.

Enzyme Assays—Uracil-DNA glycosylase inhibitor activity was measured using previously described conditions (10). When appropriate, Ugi was diluted with IDB buffer (50 \textit{mm} Tris-HCl (pH 8.0), 1 \textit{mm} EDTA, 1 \textit{mm} dithiothreitol, 100 \textit{mm} NaCl). One unit of uracil-DNA glycosylase inhibitor inactivates 1 unit of uracil-DNA glycosylase in the
standard reaction. Uracil-DNA glycosylase activity was similarly measured except that Ugi was omitted (10). One unit of uracil-DNA glyco-
sylase is defined as the amount that releases 1 mol of uracil/ h under standard reaction conditions.

**Protein Measurements**—Protein concentrations were determined by absorbance at 280 nm using the molar extinction coefficients ε_{280 nm} = 4.2 × 10^4 liter/mmol cm (Ung) and ε_{280 nm} = 1.2 × 10^4 liter/mmol cm (Ugi). The concentration of [H]Ung/Ugi complex was determined from the specific activity of [H]Ung (10).

**Electrophoresis**—Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis was performed essentially as described by Laemmli (29) and modified by Bennett et al. (12).

Nondenaturing polyacrylamide slab gel electrophoresis was performed at 4 °C essentially as described by Sanderson and Mosbaugh (19) with resolving gels containing 18% acrylamide and 0.39% N,N'-methylenebis (acrylamide). The gel was immediately stained using the rapid stain procedure described by Reiser (30) and modified by Sanderson and Mosbaugh (19).

Nondenaturing polyacrylamide tube gel electrophoresis was conducted using the same components in the resolving gel (9 × 0.6 cm diameter) and stacking gel (1 cm) as described above. Following electrophoresis the resolving gel was either stained with Coomassie Brilliant Blue G-250 or sliced horizontally into 3.1-mm sections, placed into scintillation vials, dehydrated overnight, and solubilized in 500 μl of H2O2 at 55 °C for 24–36 h as described above (12). After complete solubilization, 5 ml of Formula 989 fluor was added and 1H and 13C radioactivity was measured by scintillation spectrometry.

**Nuclear Magnetic Resonance Analysis**—All of the wild type and mutant Ugi protein samples were concentrated to 7–18 mg/ml and dialyzed against NMR buffer containing 25 mM deuterated Tris, 0.2 mM EDTA, and 100 mM NaCl, at pH 7.0. NOESY experiments were performed with 16 transients per increment. The acquisition time was 0.0832 s. There were 256 increments in t1 and 48 in t2. The data were linearly predicted to 228 points in t1 and 96 points in t2 before being Fourier-transformed into 512 × 512 × 1024 points. The spectral widths were 5000 Hz in F1, 12000 Hz in F2, and 5000 Hz for F3. These assignments led to 325 NOE constraints. A two-dimensional 13C NOESY-HMQC with a mixing time of 160 ms was collected with 16 transients per increment. There were 330 increments in t1 with the offset set in the middle of the aromatic region. Analysis of this NOE spectrum gave 35 NOE constraints. The normalized Z-score analysis of chemical shifts for Ugi produced 106 φ and ψ dihedral constraints (35).

The constraints were grouped into strong, medium, and weak. A strong NOE peak was constrained to 1.8 < r < 3.0 Å, a medium NOE peak was constrained to 2.1 < r < 4.5 Å, and a weak NOE peak was constrained to 2.4 < r < 5.0 Å during simulated annealing and refined simulated annealing protein structure determination protocols. Once the second structure of Ugi in the complex was determined there were 24 sets of hydrogen bonds that were used for a total of 48 constraints. The hydrogen bond constrained the oxygen to amide proton to be 1.8 < r < 2.5 Å and the oxygen to nitrogen distance to be 2.5 < r < 3.3 Å. The normalized Z score analysis of chemical shifts for Ugi produced 106 φ and ψ dihedral constraints (35).

The simulated annealing and refinements protocols followed the same procedures as described for the structure of the free uracil-DNA glycosylase inhibitor protein (14) as were previously reported (36). The simulated annealing and refinement protocols were run on an IBM 3CT running X-PLOR 3.1 (37).

**Protein Modeling**—Starting with the HSV-1 uracil-DNA glyco-
sylase/Ugi complex co-crystal coordinates described by Savva and Pearl (6, 17), mutant Ugi forms in complex were generated by exchanging an individual wild type Ugi amino acid with a mutant residue using the residue replacement command in INSIGHT (BIOSYM). This is thermodynamically reasonable as all the mutant Ugi structures are similar to that of the free Ugi structure as evidenced by their NOESY spectra. Complete free energy analysis of the transition from the free to the bound form of Ugi is not computationally feasible. Therefore, rigid body energy minimizations were performed to determine a reasonable esti-
mation of the energy involved between mutant forms of Ugi when bound to Ung (38). These calculations do not take into account the energy differences involved in the structural conformation changes that occur during binding to Ung. Rigid energy minimizations were then executed using an IBM 3CT running X-PLOR 3.1 (36). The rigid energy minimization procedure utilized all residues within 5 Å of the α2-helix and β1-strand of Ugi which included 40 residues of Ung and 38 residues of uracil-DNA glycosylase. A dielectric constant of 60 was used to compensate for not using water with a cut-off distance of 6.0 Å and a cutoff distance of 6.5 Å. Energy minimizations were conducted using a two-step method. The first step involved 1000 iterations of rigid energy minimization with a large van der Waals radius but without consider-
ing electrostatic forces. In the second step, 2000 iterations were con-
ducted with both electrostatic interactions and normal van der Waals radius. After the rigid energy minimization converged, the minimized structure of the uracil-DNA glycosylase-Ugi complex emerged. Each individual unbound wild type and mutant Ugi structure was similarly generated. Interaction energies were calculated by combining the van der Waals, electrostatic, and hydrogen bond energies of the enzyme-inhibitor complex and unbound Ugi. Changes in interaction energies, ΔE<sub>int</sub>, are defined as the difference in the interaction energies of the uracil-DNA glycosylase-Ugi wild type and mutant complex. The differences in the change in the interactive energies ΔE<sub>int</sub> are defined by subtracting the difference of the ΔE<sub>int</sub> of the wild type and mutant Ugi from the ΔE<sub>int</sub> of the mutant Ugi-containing complex.

**RESULTS**

**Site-directed Mutagenesis of the Uracil-DNA Glycosylase Inhibitor Gene**—To investigate the role of specific negatively charged amino acid residues in the Ung/Ugi interaction, site-
directed mutagenesis producing single amino acid substi-
tutions was performed on the ugi gene. The specific positions and substitutions were chosen due to knowledge of the 1.9-Å crystal structure of Ugi complexed with human uracil-DNA glycosylase (16). Significant similarity exists between the human and E. coli enzyme around the proposed sites of Ung/Ugi interaction (Table 1). Oligonucleotides were synthesized that introduced a codon change at seven Glu or Asp sites and a new
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**Table I**

| Ugi, amino acid | Interaction | HSV-1 | Ugi-DNA glycosylase | Human | E. coli |
|-----------------|-------------|-------|---------------------|-------|--------|
| β1-Strand       |             |       |                     |       |        |
| Glu-20          | H2          | Ser-302 | H2                 | Ser-169 | Ser-88 |
|                 | WH1         | Ser-302 |                   | Ser-270 | Ser-189 |
| α2-Helix        |             |       |                     |       |        |
| Glu-27          | H1          | Thr-280 | H2                 | Ser-247 | Ser-166 |
|                 |             | His-300 |                   | His-268 | His-187 |
| Glu-28          |             |       |                     |       |        |
|                 | H2          | Ser-305 |                   | Ser-273 | Ser-192 |
|                 | WH2         |         |                     |       |        |
| Glu-30          |             |       |                     |       |        |
|                 |             |       |                     |       |        |
| Glu-31          |             |       |                     |       |        |
| Loop regions    |             |       |                     |       |        |
| Asp-61          | H1          | Arg-252 |                   | Lys-218 | Ala-137 |
|                 |             | Pro-303 |                   | Pro-271 | Pro-190 |
|                 |             | Leu-272 |                   | Leu-191 |        |
|                 |             | Ser-273 |                   | Ser-192 |        |
|                 |             | Lys-306 |                   | Val-274 | Ala-193 |
|                 |             | WHN    |                     | Tyr-275 |        |
|                 |             |         |                     |        |        |

*a* Amino acid sequence alignment and position numbers of uracil-DNA glycosylase correspond to those described by Caradonna et al. (9). The original reference and GenBank accession number for each enzyme corresponding to HSV-1, herpes simplex virus type 1 (40) is X14112; UDG1, human (39) is X15653; and E. coli (41) is J03725.

*b* Interactions between PBS1 Ugi and HSV-1 uracil-DNA glycosylase have been described based on a 2.7-Å crystal structure of the complex (17), that involving PBS2 Ugi and human UDG1 were described from a 1.9-Å crystal structure (16).

*c* E. coli Ung amino acid residues corresponding to the aligned HSV-1 and human uracil-DNA glycosylase polypeptides are indicated (9).

*d* Chemical interactions listed include the following: H1, a hydrogen bond between carboxylate and Thr backbone amide or Arg side chains; H2, a pair of hydrogen bonds between the carboxylate and Ser backbone amide and side chain O_g; WH1, water-mediated hydrogen bond between the carboxylate and Ser-O_g; WH2, water-mediated hydrogen bonds with His backbone amide and Ser-O_g; WHN, hydrogen-bonded network with ordered solvent molecules and backbone atoms of UDG1 residues; and SB, salt bridge.

Restriction endonuclease cleavage site into the *ugi* gene as indicated in Fig. 2. To overproduce the mutant Ugi proteins, the *EcoRI/HindIII* DNA fragment containing the *ugi* structural gene was subcloned into the overexpression vector pKK223-3 producing a set of pKugi plasmids. Two methods were used to verify that the engineered mutations had been introduced into each pKugi DNA. First, restriction endonuclease digestions were conducted to establish the presence of the newly introduced recognition site within the *EcoRI/HindIII* DNA (726 bp) fragment. Second, dideoxynucleotide chain termination DNA sequencing of double-stranded pKugi DNAs was performed (data not shown). For all mutants, the entire *ugi* gene was bidirectionally sequenced and the results confirmed the designed nucleotide changes, exclusively.

**Purification and Specific Activity of the Mutant Ugi Proteins**—To facilitate characterization of the inhibitor activity exhibited by wild type Ugi and seven mutant Ugi proteins, each protein was overproduced using the appropriate pKugi vector and purified according to Sanderson and Mosbaugh (19). The purity of Ugi from the final purification step (fraction IV) was analyzed using 20% SDS-polyacrylamide gel electrophoresis (Fig. 3A). As previously observed the electrophoretic mobility of wild type Ugi was greater than that predicted for a 9474-dalton protein (10). Each mutant Ugi protein migrated with a unique slower mobility with respect to wild type Ugi, consistent with the elimination of a negatively charged residue by site-directed mutagenesis. However, these observations also imply that the mutant Ugi proteins exhibit different propensities to bind SDS or adopt to different protein conformations during electrophoresis, since each mutant protein carries the same charge.

The specific activity of each purified Ugi protein was determined under standard conditions (Fig. 3B). Ugi E20I was essentially void of inhibitory activity, displaying ~1% of the wild type specific activity, whereas Ugi E78V was virtually unaffected, displaying ~105% activity. The four mutations in Glu residues located in the α2-helix (E27A, E28L, E30L, and E31L) showed progressively decreased levels of activity with wild type 95, 88, 70, and 53% of control activity, respectively. Significant inactivation was also observed with the Ugi D61G protein which showed ~25% of wild type Ugi activity.

**Ability of Mutant Ugi Proteins to Form a Complex with Ung**—To determine whether the mutant Ugi proteins were able to form a Ung-Ugi complex, a 3-fold molar excess of Ung was incubated with each Ugi protein under standard binding conditions. The resultant Ung-Ugi complexes were resolved from the component proteins by non-denaturing polyacrylamide gel electrophoresis (Fig. 4). As controls, free Ung, wild type Ugi, and a 3:1 ratio of Ung-Ugi were analyzed for comparison with mutant forms of free Ugi and Ung-Ugi complexes. Each mutant Ugi protein migrated as a single band with a mobility similar to but slightly slower than that of wild type Ugi. In each case, the mutant Ugi proteins formed a Ugi-Ung complex that also migrated slightly slower than the wild type complex. With the exception of Ugi E20I, it appeared that each mutant Ugi protein formed a stable and complete complex with Ung since no free Ugi was detected. In contrast, the appearance of some free Ugi E20I, less Ung-Ugi E20I complex, and a smear of protein between the Ung and Ung-Ugi E20I complex bands suggested that Ugi E20I formed an unstable complex (Fig. 4, lane 5).

**Relative Ability of Mutant and Wild Type Ugi Proteins to Complex with Ung**—Competition experiments were conducted to determine the relative ability of each mutant Ugi protein to form a complex with Ung in the presence of wild type Ugi. Ung was incubated with a 2-fold molar excess of a mixture of Ugi and/or Ugi E27A at various ratios. The proteins were then resolved by non-denaturing polyacrylamide gel electrophoresis and detected by Coomassie Blue staining (Fig. 5A, lanes 1-6). Under these conditions, the Ung-Ugi E27A complexes were only partially resolved, whereas free Ugi and Ugi E27A were separated as independent bands. To quantitatively analyze the
ability of mutant Ugi proteins to compete with the wild type inhibitor protein, similar experiments were conducted after mixing each mutant Ugi with wild type \textsuperscript{35}S\textsuperscript{Ugi} and incubating the mixtures with Ung. Following electrophoresis, \textsuperscript{35}S radioactivity was detected in two bands that corresponded to \textsuperscript{35}S\textsuperscript{Ugi} free and in complex. Thus, the amount of \textsuperscript{35}S\textsuperscript{Ugi} detected in the complex band reflected the competitive ability of the mutant inhibitor protein to stably associate with Ung while in the presence of wild type Ugi. As a control, various ratios of \textsuperscript{35}S\textsuperscript{Ugi} to Ugi (both wild type proteins) were mixed and analyzed by electrophoresis (Fig. 5B, \textit{black bars}). The amount of \textsuperscript{35}S\textsuperscript{Ugi} detected in the complex approximately equaled the amount expected based on equal competition between the two inhibitor proteins and on the various ratios between \textsuperscript{35}S\textsuperscript{Ugi} and Ugi. Thus, this result confirms the identical nature of the two wild type inhibitor protein preparations and validates the experimental design. After examining each of the mutant Ugi proteins for their ability to compete with \textsuperscript{35}S\textsuperscript{Ugi} for complex formation, it was observed that two mutant Ugi proteins (Ugi E20I and Ugi E28L) consistently showed an increased amount of \textsuperscript{35}S\textsuperscript{Ugi} in complex with Ung over that predicted by equal competition. Hence, Ugi E20I and Ugi E28L demonstrated a decreased ability to form complex with Ung in the presence of wild type \textsuperscript{35}S\textsuperscript{Ugi}. The results also suggested that Ugi E20I competes very poorly with wild type Ugi since the maximum amount of \textsuperscript{35}S\textsuperscript{Ugi} based on the molar amount of Ung was found in complex for all ratios utilizing Ugi E20I.

Reversibility of the Ung-Ugi Complex with Various Mutant Ugi Proteins—To characterize further the nature of the Ung-Ugi complexes containing mutant Ugi proteins, we assessed the ability of wild type Ugi to displace mutant Ugi from a preformed complex. \textsuperscript{3}H\textsuperscript{Ung}-Ugi complexes were formed by individually incubating either wild type or mutant Ugi with a 3-fold molar excess of \textsuperscript{3}H\textsuperscript{Ung} and the complex species isolated by DEAE-cellulose chromatography. Purification of \textsuperscript{3}H\textsuperscript{Ung} was illustrated in Fig. 6A. Analysis of fractions across the peak by non-denaturing polyacrylamide gel electrophoresis verified that >95\% of \textsuperscript{3}H\textsuperscript{Ung} formed complex and that no detectable free \textsuperscript{3}H\textsuperscript{Ung} or Ugi was observed in these fractions (Fig. 6A, \textit{inset}). Stable preformed \textsuperscript{3}H\textsuperscript{Ung}-Ugi complexes were isolated using this procedure for each mutant Ugi protein (Fig. 6B) except Ugi E20I, which was unable to form a stable complex that could be purified (data not shown).

Competition experiments were conducted to determine if wild type \textsuperscript{35}S\textsuperscript{Ugi} could exchange with mutant Ugi contained in the preformed \textsuperscript{3}H\textsuperscript{Ung}-Ugi complexes. Each complex preparation was incubated with a 10-fold molar excess of \textsuperscript{35}S\textsuperscript{Ugi} and non-denaturing polyacrylamide gel electrophoresis was performed, as described above. If a mutant \textsuperscript{3}H\textsuperscript{Ung}-Ugi association was reversible, then wild type \textsuperscript{35}S\textsuperscript{Ugi} would exchange with the mutant Ugi in complex; the amount of \textsuperscript{35}S\textsuperscript{Ugi} in the complex would reflect the amount of mutant Ugi exchanged. As a control, wild type \textsuperscript{35}S\textsuperscript{Ugi} was incubated with preformed \textsuperscript{3}H\textsuperscript{Ung}-Ugi (wild type complex; 6.8\% of the \textsuperscript{3}H\textsuperscript{Ung} was found associated with \textsuperscript{35}S\textsuperscript{Ugi} in complex (Fig. 6C)). This value represents a background level when comparing results with the mutant preformed complexes. Of the six mutant Ugi contained in preformed complexes, only Ugi E28L was significantly displaced by wild type \textsuperscript{35}S\textsuperscript{Ugi} (Fig. 6C). In this case, 50\% of Ugi E28L was replaced by \textsuperscript{35}S\textsuperscript{Ugi}, demonstrating that the Ung-Ugi E28L complex was reversible. The other preformed complexes containing mutant Ugi proteins showed slightly above background levels of wild type \textsuperscript{35}S\textsuperscript{Ugi} exchange (1.9\% E27A, 0.5\% E30L, 3.4\% E31L, 1.4\% D61G, and 1.8\% E78V). Thus, in contrast to Ung-Ugi E28L the other mutant complexes...
The results indicated that rapid exchange occurred since 28\% and [35S]Ugi (6 nmol) were incubated at 25 °C for various times solubilized, and analyzed for 35S radioactivity. The average amount of wild type Ugi (control) or various mutant Ugi proteins, as indicated. The E28L complex was exploited to determine the rate of exchange to allow exchange before determining the amount of [35S]Ugi.

The reversible nature of the [3H]Ung E28L Complex—

The [3H]Ung E28L complex showed no significant exchange with [35S]Ugi after 240 min confirming the irreversible nature of this association (Fig. 7, open circles). While the Ugi E28L mutant was capable of forming a stable complex with Ung, an irreversible complex was not achieved.

Solution State Structure of Mutant and Wild Type [15N]Ugi Proteins—NMR structural determinations were made to analyze and compare the polypeptide structures of the wild type and seven mutant Ugi proteins. Each Ugi protein showed one-dimensional proton spectra consistent with a well ordered and folded structure (data not shown). The one-dimensional spectra obtained on the samples in 2H2O also indicated that all eight Ugi proteins exhibited about the same number of slowly exchanging amide protons.

Rate and Extent of Wild Type Ugi Exchange with the Ung-Ugi E28L Complex—The reversible nature of the [1H]Ung-Ugi E28L complex was exploited to determine the rate of exchange with wild type [15N]Ugi. Competition reaction mixtures containing the preformed [1H]Ung-Ugi E28L complex (0.6 nmol) and [15N]Ugi (6 nmol) were incubated at 25 °C for various times to allow exchange before determining the amount of [15N]Ugi that resided with [1H]Ung in complex (Fig. 7, closed circles). The results indicated that rapid exchange occurred since 28\% of the complex contained [15N]Ugi without incubation. The amount of exchange increased with incubation time and reached a plateau after 120 min with ~75\% of the Ugi E28L exchanging with wild type [15N]Ugi. In contrast, the [1H]Ung-Ugi (wild type) complex showed no significant exchange with [15N]Ugi after 240 min confirming the irreversible nature of this association (Fig. 7, open circles). While the Ugi E28L mutant was capable of forming a stable complex with Ung, an irreversible complex was not achieved.

Solution State Structure of Mutant and Wild Type [15N]Ugi Proteins—NMR structural determinations were made to analyze and compare the polypeptide structures of the wild type and seven mutant Ugi proteins. Each Ugi protein showed one-dimensional proton spectra consistent with a well ordered and folded structure (data not shown). The one-dimensional spectra obtained on the samples in 2H2O also indicated that all eight Ugi proteins exhibited about the same number of slowly exchanging amide protons. In addition, the distribution of the

![Image](https://example.com/image1.png)

**FIG. 5.** Ability of mutant Ugi proteins to compete with wild type [15N]Ugi for complex formation with E. coli Ung. A, six competition reaction mixtures (70 µl) containing 314 pmol of Ung and 628 pmol (total) of Ugi and/or Ugi E27A were prepared at molar ratios of 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100 ([15N]Ugi to Ugi E27A) for lanes 1–6, respectively. After the addition of Ung, the samples were mixed, incubated under standard complexing conditions, and analyzed for 3H and 35S radioactivity using a double isotope detector. B, prevention of wild type Ung-Ugi complex formation with [15N]Ugi after 240 min confirming the irreversible nature of this association (Fig. 7, open circles). While the Ugi E28L mutant was capable of forming a stable complex with Ung, an irreversible complex was not achieved.

![Image](https://example.com/image2.png)

**FIG. 6.** Ability of free [35S]Ugi to exchange with various mutant Ugi proteins in a preformed [1H]Ung-Ugi complex. A, formation and purification of the [1H]Ung-Ugi E27A complex. A sample (300 µl) containing 12 nmol of [1H]Ung and 36 nmol of Ugi E27A was incubated under standard complexing conditions and applied to a DE52 cellulose column (0.8 cm2 x 3 cm). The column was eluted with buffer A containing 150 mM and 250 mM NaCl (arrows) as described under "Experimental Procedures." Fractions (1 ml) were collected, and samples (100 µl) were analyzed for 3H radioactivity (red). Samples (40 µl) from fraction numbers 26–40 were analyzed on a nondenaturing 18% polyacrylamide slab gel (lanes 5–17). Electrophoresis was conducted at 4 °C, and protein bands were visualized after staining with Coomassie Brilliant Blue G-250 as shown in the inset. Lanes 1 contained 260 pmol of [1H]Ung; lane 2, 640 pmol of Ugi E27A; lane 3, 136 pmol of [1H]Ung-Ugi; and lane 4 contained a sample (4 µl) of the [1H]Ung/Ugi E27A mixture that was loaded onto the DE52 column. The location of the tracking dye (TD) is indicated by an arrow. Peaks fractions containing the [1H]Ung-Ugi E27A complex were pooled (bar) and concentrated using a Centriplus-10 (Amicon) concentrator. B, purity of the various [1H]Ung-Ugi complexes. Samples (40 µl) containing 100 pmol of [1H]Ung (lane B), [1H]Ung-Ugi (lanes 1 and 8), [1H]Ung-Ugi E27A (lane 2), [1H]Ung-Ugi E28L (lane 3), [1H]Ung-Ugi E30L (lane 4), [1H]Ung-Ugi E31L (lane 5), [1H]Ung-Ugi E61G (lane 6), and [1H]Ung-Ugi E78V (lane 7) were applied to a nondenaturing 18% polyacrylamide slab gel, and electrophoresis was conducted at 4 °C. The location of free Ung (arrow) and Ung-Ugi complexes (arrow) are indicated. C, ability of free [35S]Ugi to exchange with various mutant Ugi proteins in a preformed complex. Seven competition reactions (150 µl) containing 0.6 nmol of [1H]Ung-Ugi (wild type or mutant) and 6.0 nmol of [35S]Ugi (wild type) were incubated under standard complexing conditions and applied to a nondenaturing 18% polyacrylamide tube gel; electrophoresis was conducted and gels were horizontally sliced (~3 mm), dried, solubilized as indicated under "Experimental Procedures," and analyzed for 3H radioactivity using a double isotope detector. The amount of [1H]Ung ([1H]Ung) and [35S]Ugi ([35S]Ugi) found in the Ung-Ugi complex band is indicated by an arrow. Peaks reactions containing the [1H]Ung-Ugi E27A complex were pooled (bar) and concentrated using a Centriplus-10 (Amicon) concentrator.
amide proton chemical shifts was consistent with each mutant Ugi containing a high percentage of $\beta$-structure, as is the case for wild type Ugi (13, 14). Structural determinations were also made by comparing secondary structural NOE peaks from amide to amide and amide to $\alpha$-NOE spectra. The amide to amide NOESY spectra for wild type and mutant Ugi forms are shown in Fig. 8. Each mutant Ugi protein was found to contain two $\alpha$-helices and five $\beta$-strands identical to the secondary structural elements as exhibited by the unbound wild type Ugi protein. The NOESY spectra of each mutant Ugi was compared with that of the assigned wild type spectrum. Detailed examination showed that Ugi E20I, D61G, and E78V have structures that are very similar to wild type Ugi. However, the chemical shifts of many of the cross-peaks in the Ugi E20I spectrum are distinct from those of the wild type protein. Analysis of the NOESY spectra for Ugi E27A, E28L, E30L, and E31L likewise indicated close structural similarity to wild type Ugi, with the exception of the once $\alpha$-helix length. Ugi E27A and E28L contained an $\alpha$-helix that was shorter at the N-terminal end, whereas Ugi E30L and E31L exhibited a shorter $\alpha$-helix at the C-terminal end.

**Solution State Structure of $^{13}$C, $^{15}$N/Ugi Complexed to Ung**—To determine the structure of Ugi bound to Ung, a sample of $^{13}$C, $^{15}$N/Ugi (820 nmol) was combined with an excess of unlabeled Ung (1220 nmol), and the Ung$^{13}$C, $^{15}$N/Ugi complex (1.27 mM) was prepared as described previously (14). NMR structural determinations were made using a variety of NMR techniques, including 1D amide proton spectra of the wild type and mutant Ugi proteins produced by the specific amino acid replacements, NMR spectral analysis was performed for each Ugi protein. The one-dimensional proton spectra of the wild type and mutant Ugi proteins appeared to be quite similar indicating that each Ugi protein folded in much the same manner. The results of binding experiments indicated that each mutant Ugi protein remained capable of associating with Ung and forming a Ung-Ugi complex. However, complex stability and reversibility was found to be altered by some amino acid substitutions. These results suggest that none of the individual Ugi amino acids examined play an essential role in mediating Ung/Ugi binding. Rather, the negatively charged residues act collectively to facilitate stable complex formation.

The specific chemical interactions that stabilize the Ung-Ugi complex can be inferred from those in the x-ray crystallographic structures identified of HSV-1 (17) and human (16) uracil-DNA glycosylase-Ugi complexes. Such a comparison is justified since E. coli Ung shares extensive amino acid homology with its HSV-1 and human counterparts (39), and both co-crystal structures show significant structural similarity (16, 17). The structure of Ugi in complex with Ung has been determined by conventional solution state methods and found to be essentially the same as the crystal structure (16, 17). As indicated in Table I, the locations and types of interactions linking Ung residues with either HSV-1 or human uracil-DNA glycosylase were found to be highly conserved. Amino acid sequence alignment of E. coli Ung to both the HSV-1 and human enzyme revealed identical or conservative substitutions at the sites of Ugi interaction. The ability of Ugi to perform DNA mimicry has apparently capitalized on the conservation of Ung residues located in the highly conserved DNA-binding pocket (16, 18, 21). This striking amino acid correspondence suggests that similar interactions most likely mediate the Ugi association with all three uracil-DNA glycosylases examined here and possibly others.

The Ugi E20I protein, although capable of forming a Ung-Ugi complex, did not completely block Ung activity, presumably due to an inability to form a stable complex with Ung. The instability of this association was evident from the dissociation detected during nondenaturing polyacrylamide gel electrophoresis, the inability to isolate a Ung-Ugi E20I complex by anion exchange chromatography, and the ineffectiveness of Ung E20I to compete with wild type Ugi for Ung binding. The position of Glu-20 is apparently stabilized by a pair of hydrogen bonds between the carboxylate side chain of the conserved Ser-88 backbone amide and O$_2$ of E. coli Ung (Table 1). In addition, a water-mediated hydrogen bond may also form between Ung Glu-20 and Ung Ser-189, as has been described for the complex involving the HSV-1 enzyme (17). The loss of Ung E20I activity may be explained by a weakening of these interactions due to charge neutralization or peptide conformational change surrounding this key residue. Protein modeling indicated that the van der Waals energy dropped considerably (~17 kcal/mol); in this case, more than enough to stop the interaction. Taken together the results suggest that Ugi E20I forms a frail unlocked complex that fails to prevent Ung association with uracil-DNA.

The four mutations (E27A, E28L, E30L, and E31L) created
within the α2-helix had quite different effects on Ugi activity. While Ugi E27A retained near full inhibitor activity, the other three mutations caused a progressive reduction of Ugi-specific activity (E28L > E30L > E31L) with Ugi E31L maintaining ~50% wild type activity. The influence of these mutations was particularly interesting since a major structural difference between the free and complexed forms of Ugi involves the orientation of the α2-helix (Fig. 9, A and B). Furthermore, x-ray crystallographic studies have indicated that when in complex the α2-helix and β1-sheet resides over the DNA-binding groove.
and provides the majority of contacts between the enzyme and inhibitor (16, 17). Therefore, it was not surprising that Ugi E27A activity was unaffected, since Glu-27 has not been implicated in complex interaction (Table I). The results obtained for Ugi E28L and E31L support the inference drawn from chemical modification that Glu-28 and/or Glu-31 play an important role.

![Ung and Ugi Complex](image_url)

**Fig. 9. Tertiary structure of free Ugi and Ugi bound to Ung.** The tertiary structure of the solution state [15N]Ugi (A) was previously determined by Beger et al. (14). The tertiary structure of [13C,15N]Ugi bound to E. coli Ung was determined by solution state multidimensional NMR techniques as described under “Experimental Procedures.” Several secondary structural elements are highlighted in both free Ugi (A) and in complex (B) structures as follows: α1-helix (light blue); α2-helix (dark blue); β3-strand (red); β2-β5-strands (salmon); and the loop between β3- and β4-strands (yellow). The location of the N-terminal (N), C-terminal (C), Glu-28 (28), and Glu-31 (31) residues are also indicated. The electrostatic surfaces of the free (C) and complexed (D) forms of Ugi were generated using the program GRASP (38) as described previously (14). Structures A and B of the free and bound Ugi correspond to the same view as indicated in C and D, respectively. The bottom panel depicts Ugi rotated 180°. The electrostatic potentials were calculated with a dielectric constant of 6.0 for the protein and 80.0 for the solvent. The ionic strength of the solution was set to 0. Only the charges of the side chains of Lys, Asp, Asn, Glu, and Gln residues were used. The electrostatic potential cutoff was set to 6.6 kcal/mol, and the regions with a negative potential of this magnitude are shown in red, and the regions with a positive potential of this magnitude are shown in blue.
in promoting stable Ung-Ugi complex formation (19). The unique ability of Ugi E28L to form a stable but reversible complex when challenged with wild type Ugi indicates that this residue plays a critical role in forming the locked complex. Like Glu-20, Glu-28 appears to form hydrogen bonds with a conserved Ser (Ser-166 of E. coli Ung) amide and the side chain Oγ (Table I). In addition, Glu-28 also forms water-mediated hydrogen bonds to a universally conserved active site His backbone amide and Ser Oγ atom (His-187 and Ser-192 of E. coli Ung). We speculate that these contacts are responsible, at least in part, for creating the irreversible nature of the Ung-Ugi complex. Additionally, the observation that Ugi E31L, like wild type Ugi, formed an essentially irreversible complex with Ung argues that Glu-31 does not play a major role in the locking reaction.

The two mutations in the loop regions connecting the consecutive β-strands of Ugi provided distinctly different results. Ugi D61G caused a 75% reduction of activity, whereas Ugi E78V showed a specific activity equivalent to wild type Ugi. The inability of the E78V mutation to affect activity may be explained since Glu-78 resides within the electrostatic knob region of Ugi that contains seven Glu or Asp residues (14). We speculate that these contacts are responsible, at least in part, for creating the irreversible nature of the Ung-Ugi complex. Additionally, the observation that Ugi E31L, like wild type Ugi, formed an essentially irreversible complex with Ung argues that Glu-31 does not play a major role in the locking reaction.

Several lines of evidence have led to a proposal that free Ugi undergoes a conformational change during formation of the Ung-Ugi complex (14, 16, 17, 19–21). A direct demonstration of this change is evident by comparing the NMR solution structure of free [15N]Ugi with that of [15C,13N]Ugi complexed to E. coli Ung (Fig. 9). Clearly, the tertiary structure of the free and bound Ugi are quite different; however, both forms of the protein contain similar secondary structural elements (i.e., two α-helices and five β-strands). The β2-β3-β4-β5 portion of the anti-parallel β-sheet remains generally unchanged in the two structures and provides a focal point for comparison. The major transition between these structures involves a collapse of the polypeptide segments containing the α1- and α2-helices. In the unbound state, both helices extend away from the core of Ugi (14). We speculate that this Y-shaped structure may arise from the negative charge repulsion between the negative electrostatic knob and both the negatively charged α1- and α2-helices. Upon binding to Ung, the flexible arms containing the α1- and α2-helix reorient to allow the positioning of β1 and β2 over the positively charged DNA-binding pocket of uracil-DNA glycosylase (16, 17). As a consequence, several other structural changes occur as follows: (i) the β1-strand becomes twisted; (ii) the α1- and α2-helix move toward the core of Ugi; and (iii) the loop between the β3- and β4-strands becomes slightly reoriented. The orientation and negative charge of Glu-28 in the DNA-binding pocket mediates the formation of the locked complex and excludes DNA. The involvement of a Ugi structural change may explain the specificity exhibited by this inhibitor protein toward uracil-DNA glycosylases acting through a mechanism involving DNA mimicry.

The structural changes that occur during complex formation have a pronounced effect on the electrostatics of Ugi (Fig. 9). The primary changes appear to result from the positions of the α1- and α2-helices relative to the rest of the protein. The α1-helix is positioned behind the β-sheet of the complex structure shown, and the α2-helix is positioned in front. There are smaller changes of the β-strands. The helices appear to have relatively few interactions with the rest of the protein in the free form, and there may be no particularly large barriers between the free and bound conformations. The positioning in the bound state of the α1-helix effectively covers the electrostatic potential of the knob region that is exposed in the free protein. The position of the α2-helix in combination with the modest rearrangements of the β-strands gives rise to a large negative electrostatic potential on the face that forms most of the contacts in the Ung complex. This suggests that electrostatic interactions will play a considerable role in the complex and that the α2-helix appears to be involved in these interactions.

Molecular modeling studies were conducted using the co-crystal coordinates of the HSV-1 uracil-DNA glycosylase-Ugi complex and variations of the free and bound Ugi structure. The models only allowed differences in the positions of the amino acid side chains corresponding to the mutant Ugi proteins. Information concerning the contributions to the energies of complex stability was assessed for the mutations in the β1-strand and α2-helix. The modeling indicated that the complex containing Ugi E20I has by far the highest energy, consistent with the low activity of this protein. The modeling suggested that Ugi E20I forms the same unbound protein complex structure but with a smaller electrostatic potential.
structure as wild type Ugi but that there are very unfavorable (~15 kcal) van der Waals interactions in the complex. In contrast, Ugi E27A and E30L were found to have energies that are essentially identical to that of wild type Ugi in the complex. This is consistent with neither Glu-27 nor Glu-30 residues participating in an interaction with the enzyme (Table I) and both Ugi E27A and E30L showing only partially reduced activity. Models of Ugi E28L and E31L were examined in an attempt to explain the reason that Ugi E28L was the only mutant protein capable of forming a stable but reversible complex. As shown in Fig. 10, the positions of the Leu side chain should not be capable of mediating the hydrogen bond interactions that stabilize the enzyme-inhibitor complex (Table I). Under this condition Ugi E28L appears capable of conducting the docking reaction but not the locking reaction. Analysis of the energy terms showed that the electrostatics of the complexes with Ugi E28L and E31L are ~3 kcal, respectively, less favorable than that of complex containing wild type Ugi. Both mutants showed ~3 kcal less stability than wild type Ugi in complex based on van der Waals forces. Hence, Ugi E28L differed from the other mutations in the a2-helix in that it not only had the highest energy but was unfavorable in both electrostatic and van der Waals energy relative to the wild type Ugi. This suggests that the locking reaction may involve both the electrostatic potential and the hydrogen bond interactions of Glu-28.

This study has demonstrated that Ugi exists in three different conformational states (free, unlocked, and locked Ugi) during the binding reaction with Ung. The involvement of a significant structural transformation and role of Glu-20 and Glu-28 in mediating the locking reaction has been demonstrated. However, several issues remain to be investigated concerning the structure and function of Ugi during Ung complex formation. First, do individual amino acid residues play an essential role in the docking reaction? Second, what is the effect of various mutations on the kinetics of the Ung-Ugi interaction? Third, what is the structure of E. coli Ung when complexed with Ugi? Additional protein structural and biochemical analysis will be required to elucidate these important issues.

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