Identification of Specific Carboxyl Groups on Uracil-DNA Glycosylase Inhibitor Protein That Are Required for Activity*

(Received for publication, June 24, 1996, and in revised form, August 20, 1996)

Russell J. Sanderson‡§ and Dale W. Mosbaugh‡§¶

From the ‡Department of Agricultural Chemistry, the §Department of Biochemistry and Biophysics, and the ¶Environmental Health Science Center, Oregon State University, Corvallis, Oregon 97331

The bacteriophage PBS2 uracil-DNA glycosylase inhibitor (Ugi) protein inactivates uracil-DNA glycosylase (Ung) by forming an exceptionally stable protein-protein complex in which Ugi mimics electronegative and structural features of duplex DNA (Beger, R. D., Balasubramanian, S., Bennett, S. E., Mosbaugh, D. W., and Bolton, P. H. (1995) J. Biol. Chem. 270, 16840–16847; 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). The role of specific carboxylic amino acid residues in forming the Ung-Ugi complex was investigated using selective chemical modification techniques. Ugi treated with carbodiimide and glycine ethyl ester produced five discrete protein species (forms I–V) that were purified and characterized. Analysis by mass spectrometry revealed that Ugi form I escaped protein modification, and forms II–V showed increasing incremental amounts of acetyl-glycine ethyl ester adduction. Ugi forms II–V retained their ability to form a Ung-Ugi complex but exhibited a reduced ability to inactivate Escherichia coli Ung, directly reflecting the extent of modification. Competition experiments using modified forms II–V with unmodified Ugi as a competitor protein revealed that unmodified Ugi preferentially formed complex. Furthermore, unmodified Ugi and poly(U) were capable of displacing forms II–V from a preformed Ung-Ugi complex but were unable to displace Ugi form I. The primary sites of acetyl-glycine ethyl ester adduction were located in the α2-helix of Ugi at Glu-28 and Glu-31. We infer that these two negatively charged amino acids play an important role in mediating a conformational change in Ugi that precipitates the essentially irreversible Ung/Ugi interaction.

Uracil-DNA glycosylase initiates the uracil excision DNA repair pathway by removing uracil residues that accumulate in cellular DNA following dUMP incorporation or deamination of cytosine (1). The enzyme cleaves the N-glycosylic bond linking uracil to the deoxyribose phosphate backbone of DNA to produce free uracil and an apyrimidinic site (2). Uracil-DNA glycosylase is a ubiquitous and highly conserved enzyme that shares ~50% amino acid sequence homology between the proteins isolated from Escherichia coli and humans (3). Molecular modeling studies using the 1.9Å crystal structure of human uracil-DNA glycosylase revealed that uracil residues in duplex DNA must enter the catalytic pocket of the enzyme through an extrahelical rotation, termed “base flipping” (4, 5). Following uracil release, successive uracil residues may be located by a processive search mechanism (6, 7). The uracil excision DNA repair pathway of E. coli (8, 9), bovines (10), and humans (8) involves the coordinated action of uracil-DNA glycosylase, apurinic/apyrimidinic endonuclease, deoxyribophosphodiesterase, DNA polymerase, and DNA ligase to produce a one-nucleotide deletion.

Unlike most organisms that exclude uracil residues from DNA, the Bacillus subtilis bacteriophages PBS1 and PBS2 naturally exhibit ~72% base composition of U:A base pairs (11). These bacteriophage achieve stable incorporation of dUMP as a consequence of the ugi gene product, and both related phage share an identical ugi nucleotide sequence (12, 13). In vivo, the ugi gene is expressed immediately upon phage infection of the host and inactivates uracil-DNA glycosylase within 4 min postinfection, thus blocking the uracil excision DNA repair pathway (14).

The PBS2 Ugi protein has been purified to apparent homogeneity and extensively characterized as a small (9,474-dalton), monomeric, heat-stable, acidic protein of 84 amino acid residues (15, 16). The inhibitor protein contains 12 Glu and 6 Asp residues, which help to establish an acidic isoelectric point (pI = 4.2) (16). In vitro studies indicate that Ugi inactivates uracil-DNA glycosylases isolated from a wide variety of biological sources (12, 17, 18). E. coli Ung forms an extremely stable Ung-Ugi complex with 1:1 stoichiometry, which is essentially irreversible under physiological conditions (16, 19). Association of Ugi with Ung involves a two-step kinetic mechanism (19). The first step is initiated by an Ung/Ugi interaction to form a precomplex, distinguished by the dissociation constant $K_d = 1.3 \mu M$. The second step results in the formation of the final Ung/Ugi complex characterized by the rate constant $k = 195 s^{-1}$. Thus, complex formation involves a preliminary “docking” step followed by a “locking” reaction through which the two proteins achieve optimal alignment and become very tightly bound. Inhibition of Ung occurs, since the Ung/Ugi complex fails to recognize the DNA substrate (16, 19). Several observations indicate that Ugi binds to Ung at or near the DNA binding site. (i) Photochemical cross-linking of Ung to single-stranded oligonucleotide dT$_{20}$ at the DNA-binding pocket, prevented Ugi binding to Ung (20). (ii) Inclusion of Ugi in UV-induced cross-linking reactions prevented formation of the Ung × dT$_{20}$ cross-link (20). (iii) X-ray crystallographic studies of Ugi-complexed Ung.

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; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GEE, glycine ethyl ester; MALDI, matrix-assisted laser desorption-ionization; FITC, fluorescein 5-isothiocyanate; F-Ung, fluorescein 5-isothiocyanate-conjugated uracil-DNA glycosylase; PTH, phenylthiohydantoin.

* This work was supported by National Institutes of Health Grants GM32823 and ES00210. This is Technical Report 10993 from the Oregon Agricultural Experiment Station. 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.: 541-737-1797; Fax: 541-737-0497.
Uracil-DNA Glycosylase Inhibitor Protein

The tertiary structure of Ugi has been determined by solution state multidimensional nuclear magnetic resonance (24). Secondary structural elements include the α1-helix (Ser-5 to Lys-14), α2-helix (Glu-27 to Asn-35), β1-strand (Glu-20 to Met-24), β2-strand (Ile-41 to Asp-48), β3-strand (Glu-53 to Ser-60), β4-strand (Asp-69 to Asp-74), and β6 strand (Asp-79 to Leu-84). In the tertiary structure, the 12 Glu residues are shown in red and 6 Asp residues are yellow.

Several unique features of Ugi suggest that the negatively charged amino acids of the Ugi protein play an important role in mediating the Ung/Ugi interaction. First, the overall electronegative potential of the Ugi protein, engendered by its high (21%) Glu and Asp content, is quite unusual (12, 16). Second, the negative electrostatic potential of this region is >6.6 kcal, similar to that generated by the phosphate backbone of DNA (24). Fourth, both the α1 and α2 helices contain regions with similar high electronegative potential (24). Although reasons to suspect a functional role for these residues exist, the specific involvement of these negatively charged amino acids in Ung/Ugi complex formation remains to be elucidated.

In the present report, we chemically modify the carboxylic acid residues of Ugi to determine their role in forming the Ung/Ugi complex. Specifically, the approach involved (i) chemical modification of Ugi with the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and the nucleophile glycine ethyl ester (GEE); (ii) purification of five modified forms of Ugi; (iii) characterization of each form concerning the effect of modification on Ugi specific activity, Ung/Ugi complex stability, and reversibility; and (iv) identification of specific sites of Ugi addition using matrix-assisted laser desorption-ionization (MALDI) mass spectrometry and amino acid sequencing techniques. The findings establish the importance of two Glu residues in the α2-helix for achieving stable Ung/Ugi complex formation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fluorescein 5-isothiocyanate (FITC isomer I) was purchased from Molecular Probes, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and glycine ethyl ester were from Sigma. Isopropyl-1-thio-β-D-galactopyranoside came from Life Technologies, Inc., cyanogen bromide was obtained from Aldrich, and endoproteinase Asp-N was from Boehringer Mannheim. [35S]Methionine was purchased from DuPont-NEN and [3H]dUTP from Amersham Corp. Calf thymus uracil-(H)DNA from Life Technologies, Inc., was used in mediating the Ung/Ugi interaction.

The tertiary structure of bacteriophage PBS2 uracil-DNA glycosylase inhibitor protein. The tertiary structure of Ugi has been determined by solution state multidimensional nuclear magnetic resonance (24). Secondary structural elements include the α1-helix (Ser-5 to Lys-14), α2-helix (Glu-27 to Asn-35), β1-strand (Glu-20 to Met-24), β2-strand (Ile-41 to Asp-48), β3-strand (Glu-53 to Ser-60), β4-strand (Asp-69 to Asp-74), and β6 strand (Asp-79 to Leu-84). In the tertiary structure, the 12 Glu residues are shown in red and 6 Asp residues are yellow.
following modifications: (i) E. coli JM105 cells transformed with pTZ57R in 9 liters of LB medium at 37°C for 24 h, and (ii) urea-PAGE slab gels were placed in dye solution containing 0.04% (w/v) Coomassie Brilliant Blue G-250 and 3.5% (v/v) HClO4 immediately after electrophoresis. Complete staining required gentle agitation at 25°C for 12 h. Gels were destained with 5% (v/v) acetic acid, and the gel was visualized with Polaroid-55 film using an orange filter (Eastman Kodak Co.; 23A).

Nondenaturing polyacrylamide tube gel electrophoresis was performed as described by Davis (29) with some modifications. Tube gels (0.6-cm diameter) contained a resolving gel (9 cm) and stacking gel (1 cm). Protein samples were prepared, and electrophoresis was performed at 4°C as described above. To detect [%35S]Ugi and Ung [%35S]Ugi, gels were sliced horizontally into 3.1-mm sections, placed into scintillation vials (7 ml), and dehydrated overnight at room temperature. For Ung [%35S]Ugi, the addition of 100% DMSO was stopped and incubated at 55°C for 36–48 h. Once solubilized, 5 ml of Formula 989 (DuPont) fluoro was added, and %35S radioactivity was measured in a Beckman LS 8600 liquid scintillation counter.

Fluorescein 5-Isothiocyanate Labeling of Ung—Ung (fraction V) was extensively dialyzed against buffer KEG containing 50 mM potassium phosphate (pH 9.5), 1 mM EDTA, and 5% (w/v) glycerol at 4°C. Fluorescein 5-isothiocyanate conjugate was freshly prepared in dimethylformamide, and 1.5 ml was added to 14 ml of Ung (2.9 mg) with thorough mixing. After 2.5 h at 25°C in the dark, the mixture was placed on ice and then loaded onto a Sephadex G-25 column (4.9 cm × 13.5 cm) equilibrated in buffer KEG. Fractions (2 ml) were collected, and samples were monitored at 280 and 496 nm for absorbance. After confirming unity of fluorescence, FITC-UNG concentration was determined based on absorbance at 280 and 496 nm. The concentration of FITC in the dye-protein conjugate (2.3 fluorophores/enzyme molecule) was determined by the absorbance, where e280nm = 7.8 × 104 liters/mole/cm (30). It was experimentally determined that 1 µM FITC contributed 0.025 A280. Thus, the concentration of F-Ung was determined by subtracting the A280 contributed by FITC from the overall A280 of the dye-protein conjugate and dividing by the molar extinction coefficient of Ung. No significant alteration of Ung activity was detected following production of F-Ung (19).

Fluorescence Measurements—Steady-state fluorescence measurements were conducted at 25°C using an LS50 luminescence spectrometer (Perkin-Elmer) equipped with a xenon flash tube and a thermoelectron microtiter (4 mm). Excitation and emission wavelengths were 496 and 520 nm, respectively; both slit widths were set at 5 mm. Cyanogen Bromide Cleavage of Ugi Protein—Cyanogen bromide cleavage reaction mixtures contained Asp-N (0.05 mg/ml in 10 mM Tris-HCl (pH 7.5)) and [35S]Ugi or EDC/GEE-modified [%35S]Ugi forms I–V. After incubation at 25°C for 24 h, samples were evaporated to dryness, resuspended in 600 µl of distilled water, and subjected to mass spectrometric methods. Samples utilized for peptide C3 purification were resuspended in 600 µl of buffer A and applied to individual DE-52 cellulose column (0.79 cm × 1.3 cm) equilibrated in buffer A at 4°C. After sample loading, the flow rate was stopped for 5 min, and then the column was washed with 2.5 ml of buffer A. Peptide fragments were eluted with a 50-mL linear gradient of 0–250 mM NaCl in buffer A. Fractions (500 µl) were collected in Eppendorf tubes, and samples (1 µl) were analyzed for CNBr-cleaved peptide fragments by MALDI mass spectrometry. Fractions containing peptide C3 were pooled and concentrated to ~300 µl using a Centricon-3 (Amicon) concentrator, buffer was exchanged with distilled water, and fractions were submitted for amino acid sequencing analysis.

Asp-N Endopeptidase Digestion of Ugi Protein—Proteolysis reaction mixtures contained Asp-N (0.04 mg/ml in 10 mM Tris-HCl (pH 7.5)) and [%35S]Ugi or EDC/GEE-modified [%35S]Ugi forms II–III (10 mM Tris-HCl (pH 7.5)) in a 1:100 (w/w) ratio. Incubation was carried out for 3 h at 25°C, and the extent of proteolysis was monitored by MALDI mass spectrometry.

Mass Spectrometry—MALDI mass spectrometry was performed by the Mass Spectrometry Facilities and Service Core Unit (Environmental Health Science Center, Oregon State University) using a custom-built time of flight instrument (31). Two different matrices were used in the sample preparation: (i) 10 µg/ml of sinapinic acid dissolved in 33% acetonitrile and 67% trifluoroacetic acid (0.1% solution) was mixed (3:1) with samples (0.5 µl) containing 10 µM modified or unmodified Ung protein; and (ii) a saturated solution of α-cyano-4-hydroxycinnamic acid in 33% acetonitrile and 67% trifluoroacetic acid (0.1% solution) was mixed (10:1) with samples (0.5 µl) containing CNBr-treated Ung purified peptide C3, and Asp-N-digested Ung protein. Each matrix solution was applied to a mass spectrometric probe and allowed to dry as described previously by Bennett et al. (20). A mass spectrum was...
Ugifromtheunfractionatedmodificationreactionmixture.
Theotherproteinbands (II–V) observed in the reaction mix-
ture was then applied to a DE-52 cellulose column equilibrated in the same
buffer. The column was washed and eluted with a linear gradient of 50–300
mM NaCl in buffer A, fractions (1 ml) were collected, and samples were monitored
for conductivity (●) and 35S radioactivity (○). Fractions were pooled corresponding to
the various forms (I–V), as indicated by brackets, and evaporated to dryness; each
pool was resuspended in buffer A (1 ml) and dialyzed against buffer EB.

generated from 30 individual laser pulses (+24 kV), and the summed
signals were calibrated using standard ion signals from the matrix.

Amino Acid Sequencing—Samples (30 μl in H2O) containing purified
peptide C3 from Ugi or modified Ugi forms I–IV were applied to a
cartridge filter precycled with Biobrene (Applied Biosystems). Amino
acid sequencing was conducted using an Applied Biosystems model
gas phase protein sequencer by the Center for Gene Research and
Biotechnology (Oregon State University).

RESULTS

Modification of Carboxylic Acid Residues in Uracil-DNA Glycosylase Inhibitor Protein—To assess the importance of Glu
and Asp residues in mediating the Ung/Ugi interaction, chemical
modification of Ugi was conducted, and the effect on inhibitor
activity was investigated. The water-soluble carbodiimide,
EDC, was used in a two-stage modification reaction with GEE
to selectively adduct carboxyl groups (32). Under mild reaction
conditions, this carbodiimide preferentially forms an O-acetyl-
sourea-activated carboxyl group that subsequently undergoes
nucleophilic attack by GEE, forming a terminal acyl-glycine
ethyl ester reaction product (32). A limited modification reac-
tion was conducted, and modified forms of 35S[Ugi] were re-
solved by DEAE-cellulose chromatography (Fig. 2). Five dis-

tinct forms (I–V) were identified eluting at about 180, 165, 150,
140, and 140 mM NaCl, respectively.

Identification and Purity of Ugi Forms I–V—The five
35S[Ugi] peaks were separately pooled, and each Ugi form was
analyzed by nondenaturing polyacrylamide gel electrophoresis
(Fig. 3, lanes 4–8). Samples of unmodified 35S[Ugi (lane 1),
35S[Ugi from the unfraccionated modification reaction mixture
(lane 2) and mock-modified 35S[Ugi (lane 3) were analyzed for
comparative purposes. The unfraccionated 35S[Ugi sample
contained five protein bands; three were very discrete (bands
I–III), and two appeared to be more diffuse (bands IV and V).

Band I corresponded to unmodified Ugi (lane 1) which comi-
grated with the single protein band of 35S[Ugi form I (lane 4).
The other protein bands (II–V) observed in the reaction mix-
ture (lane 2) were each individually represented by 35S[Ugi
forms II–V, respectively (lanes 5–8). Forms II–V showed pro-
gressively reduced electrophoretic mobility that inversely cor-
related with their order of elution from the DEAE-cellulose
column (Fig. 2). Taken together, these results indicate that
EDC/GEE-mediated modification of Ugi (forms II–V) caused
negative charge neutralization of carboxyl groups.

The purity of 35S[Ugi forms I–V was assessed based on the
relative Coomassie Brilliant Blue staining band intensities of
individual fractions from across the DEAE-cellulose column
(data not shown) and from the radioactivity of 35S[Ugi de-
tected in gel slices of forms I–V (Fig. 3). These results indicated
that forms I–III are ≥90% pure, whereas forms IV and V appear to contain ≥65% of the corresponding form. From the
35S radioactivity detected in gel slices of forms I–V (Fig. 3, lane
2), it was determined that forms I–V constituted approximately
14, 23, 26, 18, and 10% of the total 35S[Ugi, respectively.

To determine the extent of EDC/GEE-mediated modification,
each Ugi form along with unmodified Ugi was analyzed by
MALDI mass spectrometry (Table I). As previously observed
(16), two mass peaks were obtained for the unmodified Ugi
protein: peak I (9,475 daltons) closely agreed with the predicted
mass of the complete amino acid sequence of Ugi, and peak II
(9,341 daltons) corresponded with Ugi protein minus the N-
terminal methionine. Ugi form I also contained two species with masses nearly identical to those of the unmodified Ugi control and apparently escaped modification during the EDC/GEE reaction. Ugi form II appeared to contain a single acyl-glycine ethyl ester modification, since this adduct would be expected to add 85 daltons per modified carboxyl group. By dividing the mass increase observed for each modified Ugi form by 85 daltons, it was deduced that forms II–V contained approximately 1.1, 2.0, 2.9, and 3.8 adducts, respectively, per Ugi protein.

**Effect of EDC/GEE Modification on the Activity of Ugi Forms I–V.**—The effect of chemical modification on inhibitor activity of purified Ugi forms I–V was determined using *E. coli* uracil-DNA glycosylase (Fig. 3). The specific activity of unmodified Ugi was essentially the same as either the mock EDC/GEE-modified Ugi or Ugi form I. The slight increase in specific activity observed for the modified protein sample may have resulted from the removal of inactivated Ugi contained in the original preparation, during DEAE-cellulose chromatography. In contrast, the specific activity of Ugi forms II–V displayed progressively decreased levels (58–17%) of inhibitor activity coinciding with the increased extent of Ugi modification.

**Ability of Ugi Forms I–V to Bind Ung—**Ugi forms I–V were incubated with a 3-fold molar excess of Ung under conditions that typically promote Ung-Ugi complex formation. The complex was then resolved from its individual components by non-denaturing polyacrylamide gel electrophoresis. As controls, unmodified [35S]Ugi, EDC/GEE-modified [35S]Ugi reaction mixture, and Ung were individually separated by electrophoresis (Fig. 4, lanes 1–3). The addition of unmodified Ugi or EDC/GEE-modified Ugi reaction mixture to excess Ung resulted in Ung-[35S]Ugi complex formation of 99 and 75% of [35S]Ugi, respectively (Fig. 4, lanes 4 and 5). Interestingly, the Ung-Ugi complex formed by Ugi from the EDC/GEE reaction mixture resulted in a series of bands with decreased mobility (lane 5). When individual Ugi forms I–V were analyzed, the uncomplexed [35S]Ugi bands disappeared coincident with the appearance of 97, 90, 77, 67, and 61% of the [35S]Ugi in complex with Ung (lanes 6–10). The reduced electrophoretic mobility of each complex was explained by the decreased electronegativity of individual EDC/GEE-modified Ugi forms. These findings demonstrate that modified Ugi forms remain capable of forming a Ung-Ugi complex.

**Competitive Interaction between Ugi and EDC/GEE-modified Ugi Forms I–V for Ung Binding—**Experiments were conducted to determine the competitive ability of unmodified Ugi and EDC/GEE-modified [35S]Ugi forms I–V to form a stable complex with Ung. In the first experiment, unlabeled Ugi was separately mixed with each of the modified [35S]Ugi forms in various molar ratios (100:0, 80:20, 60:40, 40:60, and 20:80; modified:unmodified Ugi). Each Ugi mixture was combined with Ung in a 2-fold molar excess of inhibitor over enzyme to form complex, and the proteins were resolved by non-denaturing polyacrylamide gel electrophoresis. Samples containing various ratios of unmodified Ugi competitor were mixed with unmodified [35S]Ugi, as controls, and analyzed by electrophoresis (Fig. 5A). Two peaks of [35S]Ugi were observed in each

---

**TABLE I**

Acyl-glycine ethyl ester adducts detected on EDC/GEE-modified Ugi using MALDI mass spectrometry

| Ugi        | Predicted mass (A) | Experimental mass (B) | Mass increase (B – A) | Acyl-GEE adducts/Ugi |
|------------|--------------------|-----------------------|-----------------------|----------------------|
| Unmodified |                    |                       |                       |                      |
| Peak I     | 9,477              | 9,475 ± 10.5          | 1                     | 0                    |
| Peak II    | 9,346              | 9,341 ± 3.0           | 0                     | 0                    |
| Form I     |                    |                       |                       |                      |
| Peak I     | 9,477              | 9,478 ± 8.1           | 1.1                   | 1.1                  |
| Peak II    | 9,346              | 9,346 ± 0.1           | 0                     | 0                    |
| Form II    |                    |                       |                       |                      |
| Peak I     | 9,477              | 9,569 ± 4.7           | 92                    | 92                   |
| Peak II    | 9,346              | 9,437 ± 0.6           | 91                    | 91                   |
| Form III   |                    |                       |                       |                      |
| Peak I     | 9,477              | 9,646 ± 8.4           | 169                   | 169                  |
| Peak II    | 9,346              | 9,507 ± 0.1           | 161                   | 161                  |
| Form IV    |                    |                       |                       |                      |
| Peak I     | 9,477              | 9,727 ± 0.8           | 250                   | 250                  |
| Peak II    | 9,346              | 9,594 ± 0.7           | 248                   | 248                  |
| Form V     |                    |                       |                       |                      |
| Peak I     | 9,477              | 9,794 ± 0.5           | 317                   | 317                  |
| Peak II    | 9,346              | 9,665 ± 5.7           | 319                   | 319                  |

a Predicted mass values were determined by the computer program MacProMass, version 1.05, by Lee and Vermuri (38).
b Average molecular weights and standard errors are determined from two independent measurements.
c One acyl-glycine ethyl ester adduct per Ugi protein would be expected to result in an 85-dalton mass increase.
d Peak I corresponds to the intact Ugi protein.
e Peak II corresponds to the Ugi protein minus 131 daltons, which correlates to the loss of the amino-terminal methionine residue (16).
sample, one corresponding to unbound [35S]Ugi and the other to the Ung-[35S]Ugi complex. As expected, the percentage of [35S]Ugi forming a complex almost exactly reflected the ratio of [35S]Ugi to unmodified Ugi in the unmodified reaction mixture (Fig. 5B, inset). Similar results were obtained when [35S]Ugi forms I–V were used with unlabelled native Ugi as the competitor. The amount of [35S]Ugi (in pmol) forming complex in the absence of competitor (100:0) is given in parenthesis in the inset. The relative percentage of unmodified or modified (forms I–V) [35S]Ugi that forms a complex during the competition reactions relative to the 100:0 control was determined for each ratio of [35S]Ugi to unmodified Ugi. Each reaction was conducted in duplicate, and percentages of [35S]Ugi complexed represent averaged values.

Stability of the Ung-Ugi Complex Containing EDC/GEE-modified Ugi Forms I–V—To examine the complex stability of modified Ugi forms I–V, 5.3 nmol of either unmodified or modified forms (I–V) of [35S]Ugi were incubated with a 5-fold molar excess of Ung, and the Ung-[35S]Ugi complex was purified from uncomplexed proteins by DEAE-cellulose chromatography (Fig. 6A). In each case, essentially all of the [35S]Ugi eluted as Ung-[35S]Ugi complex following the 150 mM NaCl step. No significant amount of free inhibitor protein was detected after the 250 mM NaCl elution step, and excess Ung eluted in the wash fractions. Each Ung-[35S]Ugi complex preparation was then analyzed by nondenaturing polyacrylamide gel electrophoresis to assess complex formation and stability (Fig. 6B). Purified Ung-[35S]Ugi complexes containing unmodified form I and II [35S]Ugi each contained >95% of the total inhibitor protein in complex (Fig. 6C). In contrast, free [35S]Ugi was observed for Ugi forms III–V in complex with Ung, as demonstrated by 17, 34, and 30% of the total [35S]Ugi migrating as a smear of uncomplexed inhibitor protein, respectively. A diffuse band of free Ung also appeared in these samples. These results were consistent with an interpretation that more extensive levels of EDC/GEE modification of Ugi cause less stable complexes to dissociate during electrophoresis.

To determine if modified forms I–V of [35S]Ugi in complex could exchange with free Ung, each complex preparation was incubated with a 3- and 30-fold molar excess of unmodified Ugi, and gel electrophoresis was performed, as before, to resolve the constitutive components (Fig. 6, D and E). As anticipated for the control Ung-[35S]Ugi, no significant release of unmodified

FIG. 5. Ability of unmodified Ugi to compete with modified [35S]Ugi forms I–V for complex formation with Ung. A, competition reaction mixtures (70 μl) contained 158 pmol of Ung and 316 pmol combinations of unmodified [35S]Ugi and unlabeled competitor Ugi at [35S]Ugi:Ugi ratios of 100:0 (●), 80:20 (■), 60:40 (■), 40:60 (□), and 20:80 (▲). Following Ung addition, the reaction mixtures were incubated under standard complexing conditions and then loaded onto 18% non-denaturing polyacrylamide tube gels as described under “Experimental Procedures.” After electrophoresis, each gel was horizontally sliced (3.1 mm), dried overnight, solubilized in 30% H2O2 (500 μl), and analyzed for 35S radioactivity. The direction of migration was from left to right, and the location of the tracking dye (TD) is indicated by an arrow. B, radioactive peaks containing Ung-[35S]Ugi and unbound [35S]Ugi were measured for the unmodified [35S]Ugi control and competition reactions at various ratios with competing unmodified Ugi (■). Competition reactions as described in A were repeated, but [35S]Ugi forms I (■), II (■), III (■), IV (▲), and V (□) were used with unlabelled native Ugi as the competitor. The amount of [35S]Ugi (in pmol) forming complex in the absence of competitor (100:0) is given in parenthesis in the inset. The relative percentage of unmodified or modified (forms I–V) [35S]Ugi that forms a complex during the competition reactions relative to the 100:0 control was determined for each ratio of [35S]Ugi to unmodified Ugi. Each reaction was conducted in duplicate, and percentages of [35S]Ugi complexed represent averaged values.
unmodified[Ugi(35S)] (85 pmol of purified complex formed with above. Complex was carried out as described for modified forms(I–V) of [35S]Ugi. Purification of each corresponding Ung complex containing Ung and each of the unmodified [35S]Ugiforms (I–V) was determined under standard assay conditions. The preformed complex containing unmodified [35S]Ugi each containing 228 pmol of preformed Ung-[35S]Ugi complex was constituted with unmodified [35S]Ugi (lane 1) and modified [35S]Ugi forms I–V (lanes 2–6, respectively) were incubated using 18% nondenaturing polyacrylamide gel electrophoresis. Protein was visualized after staining with Coomassie Brilliant Blue G-250; the arrow indicates the location of Ung, and the bracket denotes the Ung-[35S]Ugi complex. C–E, ability of free Ugi to exchange with the various forms of [35S]Ugi in complex. Competition reaction mixtures (140 μl) each containing 228 pmol of preformed Ung-[35S]Ugi complex constituted with unmodified [35S]Ugi (lane 1), II (3), III (4), IV (5), or V (6) were incubated with buffer (C), 684 pmol of Ugi (D), or 6,840 pmol of Ugi (E) under standard complex formation conditions. Samples were loaded onto 18% nondenaturing polyacrylamide tube gels; electrophoresis was performed; and gels were sliced, solubilized, and analyzed for [35S] radioactivity as described under “Experimental Procedures.” Fractions (1 ml) were collected and analyzed for 35S-labeled protein. Fractions (nos, 33–44) containing the Ung-[35S]Ugi complex were pooled and concentrated (∼12-fold). Five similar reaction mixtures were prepared containing Ung and each of the modified forms (I–V) of [35S]Ugi. Purification of each corresponding Ung-[35S]Ugi complex was carried out as described above. B, nondenaturing polyacrylamide gel analysis of the isolated Ung-[35S]Ugi complexes. Six samples (50 μl) containing 85 pmol of purified complex formed with unmodified [35S]Ugi (lane 1) and modified [35S]Ugi forms I–V (lanes 2–6, respectively) were analyzed using 18% nondenaturing polyacrylamide gel electrophoresis. Protein was visualized after staining with Coomassie Brilliant Blue G-250; the arrow indicates the location of Ung, and the bracket denotes the Ung-[35S]Ugi complex.

[Ugi(35S)] (<2.5%) was detected, indicating the irreversibility of the complex. A nearly identical result was obtained for Ung-[35S]Ugi form I complex. In contrast, the presence of a 3-fold molar excess of Ugi promoted the release of 27, 50, 63, and 54% of total modified Ugi forms II–V from the complex, respectively (Fig. 6D). Similar increases in the amount of [35S]Ugi released were observed for the exchange reactions containing the 30-fold molar excess of Ugi (Fig. 6E). These results further suggest that charge neutralization due to modification leads to the destabilization of the irreversible Ung-Ugi complex.

If modification of Ugi facilitated Ung-Ugi dissociation in the presence of exogenous Ugi, then perhaps these complexes also undergo dissociation in the absence of competing Ugi. Free Ung produced from a dissociated complex might be detected by measuring uracil-DNA glycosylase activity. Therefore, the enzymatic activity of Ung in each complex containing unmodified or modified Ugi forms I–V was determined under standard assay conditions. The preformed complex containing unmodified Ugi showed <0.1% of the total uracil-DNA glycosylase in complex displaying catalytic activity. In contrast, Ung complexed with Ugi forms I–V, respectively, possessed 2, 18, 46, 37, and 28% of the uracil-DNA glycosylase activity expected for completely uncomplexed and uninhibited Ung. Taken together, these results confirm that EDC/GEE-modified forms of Ugi are less capable of maintaining an irreversible and catalytically inactive Ung-Ugi complex.

Steady-state Fluorescence Measurements of Fluorescein 5-Isothiocyanate-labeled Ung Binding to Ugi Forms I–V—To further investigate the properties of EDC/GEE-modified Ugi interactions with Ung, the enzyme was labeled with FITC to produce F-Ung. As previously reported (19), F-Ung fluoresces, when quenched, function as reporter groups for both Ugi and nucleic acid binding. To determine whether fluorescence quenching was quantitative between differentially modified forms of Ugi, F-Ung was titrated with Ugi, and the relative fluorescence was monitored under steady-state conditions (Fig. 7). In the control, the addition of unmodified Ugi caused a proportional decrease in fluorescent signal and elicited a 9.4% maximal quench achieved at a Ugi:Ung molar ratio of 8:1. Titration of F-Ung with modified Ugi forms I–V also displayed a linear decrease in fluorescence intensity with maximum flu-
Uracil-DNA Glycosylase Inhibitor Protein

Effect of Ugi Forms I–V on F-Ung Binding to Poly(U) — The linearity of the titration curves for Ugi forms I–V binding to F-Ung (Fig. 7) indicate that Ugi remains in complex with uracil-DNA glycosylase after complex formation and does not freely dissociate. This observation would appear to contradict findings showing that free Ugi will exchange with modified forms II–V in the preformed complex and that Ung demonstrates catalytic activity in these same complexes. However, an explanation for both results could be that complexes are maintained in solution but that the addition of competitor Ugi or nucleic acid present in the standard reaction mixture promotes dissociation of modified complexes. Thus, the influence of Ugi on F-Ung binding to poly(U) was investigated. When F-Ung (42 pmol) was combined with a saturating amount of poly(U), the average fluorescence intensity was quenched by 7.9 ± 0.8% (Fig. 8A, ΔRF1). The addition of unmodified [35S]Ugi (63 pmol) resulted in a further fluorescence intensity decrease of 4.5% (Fig. 8A, ΔRF2), indicating that Ugi preferentially binds to F-Ung in the presence of poly(U) and effectively competes poly(U) out of the complex. Similar experiments were conducted using modified Ugi forms I–V in place of the unmodified Ugi. After the initial fluorescence quench (ΔRF1, ~8%) due to poly(U), the addition of Ugi forms I–V resulted in ΔRF2 values of 4.3, 3.1, 1.4, 1.2, and 2.1%, respectively (Fig. 8B). Each of the EDC/GEE-modified Ugi forms, except Ugi form I, showed a significantly reduced ΔRF2 value compared with the unmodified Ugi control (ΔRF2 = 4.5%).

As a second approach, an analogous experiment was conducted where F-Ung (42 pmol) was combined with either unmodified or modified Ugi forms I–V (63 pmol) prior to poly(U) exposure, and the relative fluorescence intensity was monitored after each addition to determine ΔRF1 and ΔRF2 (Fig. 8C). The initial addition of unmodified Ugi caused a 8.5% reduction of fluorescence intensity, and no further quenching of the fluorescent signal was observed following the poly(U) addition (ΔRF2 < 0.17). This observation was consistent with an interpretation that the Ung-Ugi complex was refractory to binding nucleic acid (19). Conversely, the addition of poly(U) to each of the EDC/GEE-modified forms of Ugi in complex with...
F-Ung elicited additional fluorescence quenching (Fig. 8C, D). RF2). Collectively, these results demonstrate the reversibility of modified Ugi forms in complex with Ung when nucleic acid is present, a property not exhibited by the unmodified Ugi–Ung complex.

Identification of EDC/GEE-modified Amino Acid Residues in Ugi—In order to locate EDC/GEE-modified residues of Ugi forms I–V, the inhibitor protein was chemically cleaved with cyanogen bromide, and the peptide fragments were analyzed by MALDI mass spectrometry (Figs. 9 and 10). Ugi contains four methionine residues capable of producing five peptide fragments following complete CNBr-induced cleavage (Fig. 9). Four peptide fragments were identified for unmodified Ugi; mass values were in excellent agreement with the predicted mass of peptides C1/2, C2, C3, and C4 (Table II). A similar analysis was performed on EDC/GEE-modified Ugi forms I–V (Fig. 10 and Table II). As anticipated, Ugi form I generated a set of four peptides with nearly the same mass values as the unmodified Ugi control. In contrast, five peptide fragments were identified for CNBr-treated Ugi form II; four corresponded to unmodified peptides C1/2, C2, C3, and C4. The fifth peptide (C3M1), comprising 82% of C3-derived peptide fragments, had a mass of 3,643 daltons, which was 89 daltons larger than that predicted for peptide C3. These findings indicate that the major site of adduction is located on the C3 peptide. Similarly, the vast majority (92%) of Ugi form III adducts were localized on the C3 peptide (Table II). A second modified peptide (C3M2) was identified with a mass of 3,638 daltons, indicative of two adducts, each of ~85 daltons. Ugi form III also produced a C3M3 peptide fragment (3,638 daltons), connoting a single acyl-glycine ethyl adduction (Table II). Mass determination of CNBr-treated Ugi forms IV and V both included peptide fragments C3M1 and C3M2 and possessed additional peptides C3M3 and C3M4. The relative percentage of peptides C3M1–4 detected for each Ugi form is indicated in Table II. In all cases, the primary modification site(s) were specifically localized to peptide C3, which contained 10 Glu and Asp residues.

The location of the adducted site(s) on peptide C3 was further investigated using the same approach but following endoprotease Asp-N digestion of Ugi. A comparison of the molecular weights of unmodified and form II Ugi peptides revealed two modified fragments derived from peptides A2 and A6/7 (Table III). The major A2M1 (~82%) and minor A6/7M1 (~23%) modified peptide species both showed a mass increase of ~85 daltons, denoting the presence of a single acyl-glycine ethyl ester adduct. Peptide A2M1 was detected in equal abundance with C3M1 and shared overlapping amino acid sequence from Leu-25 to Ser-39 (Fig. 9). Thus, we deduced that modification by EDC/GEE likely involved Glu-27, Glu-28, Glu-30, Glu-31, and/or Glu-38. The appearance of peptide A6/7M1 suggested that another site of adduction occurred with reduced frequency at Glu-64, Asp-74, Glu-78, and/or Glu-83. The appearance of peptide A6/7M1 suggested that another site of adduction occurred with reduced frequency at Glu-64, Asp-74, Glu-78, and/or Glu-83.

2 CNBr peptide fragments C1 and C5 correspond to the single amino acids, methionine and leucine, that were not detected by MALDI mass spectrometry.
Ugi and Ugi forms I–V in Fig. 10. Molecular mass of Ugi peptides produced by endoproteinase Asp-N

| Peptide | Predicted mass\(a\) | Experimental mass\(b\) |
|---------|----------------------|------------------------|
|         | Unmodified Form I Form II Form III Form IV Form V |
|         | daltons              | daltons                |
| C1/2    | 2,674 (38)           | 2,671 (37)            |
| C2      | 2,554 (38)           | 2,556 (38)            |
| C3      | 3,554 (38)           | 3,558 (38)            |
| C3M1    | 3,643 (89)\(d\)      | 3,638 (84)            |
| C3M2    | 3,710 (156)          | 3,712 (158)           |
| C3M3    | 3,795 (241)          | 3,792 (238)           |
| C3M4    | 3,846 (310)          | 3,840 (86)            |
| C4      | 3,012 (38)           | 3,015 (38)            |

Relative amounts of C3 peptides\(e\)

| C3     | >95 | >95 | 18 | 8 | 5 | 7 |
|--------|-----|-----|----|---|---|---|
| C3M1   | 82  | 22  | 17 | 12|
| C3M2   | 70  | 34  | 29 |
| C3M3   | 28  | 22  | 25 |
| C3M4   | 16  | 16  | 27 |

* Predicted mass values of CNBr-generated peptides were determined by the computer program MacProMass, version 1.05, by Lee and Vermuri (38).

\(d\) Average molecular masses were determined from four independent measurements, and standard errors range from 0.3 to 4.5 daltons.

* Peptide C1/2 contains the amino-terminal methionine residue that was neither post-translationally removed in vitro (39) nor cleaved by CNBr to generate peptide C2 in vitro, possibly due to oxidation of the methionine to the sulfone.

* Numbers in parentheses correspond to mass values obtained by subtracting the experimentally determined mass from the predicted mass and reflect mass increases.

* Relative amounts of modified or unmodified peptide C3 were determined as percentages of total peak area (3,500–4,000 daltons) for unmodified Ugi and Ugi forms I–V in Fig. 10.

| Peptide | Predicted mass\(a\) | Experimental mass\(b\) |
|---------|----------------------|------------------------|
|         | Unmodified Form II Form III |
|         | daltons              | daltons                |
| A1\(a\) | 565 (95)             | 565 (95)               |
| A2      | 3,837 (95)           | 3,838 (95)            |
| A2M1    | 3,920 (83)\(d\)      | 3,929 (86)            |
| A2M2    | 3,999 (182)          | 3,999 (182)           |
| A3      | 932 (95)             | 932 (95)              |
| A4\(a\) | 451 (95)             | 451 (95)              |
| A5\(a\) | 1,021 (95)           | 1,021 (95)            |
| A6\(a\) | 1,530 (95)           | 1,530 (95)            |
| A6/7\(a\)| 2,759 (95)       | 2,760 (95)            |
| A6/7M1  | 2,846 (87)           | 2,844 (85)            |
| A7      | 1,249 (95)           | 1,249 (95)            |

Relative amounts of A2M\(a\)

| A2     | 18  | 10  |
|--------|-----|-----|
| A2M1   | 82  | 25  |
| A2M2   | 65  |     |
| A6/7   | 77  | 63  |
| A6/7M1 | 23  | 37  |

* Predicted mass values of CNBr-generated peptides were determined by the computer program MacProMass, version 1.05, by Lee and Vermuri (38).

\(d\) Average molecular masses were determined from four independent measurements, and standard errors range from 0.0 to 4.5 daltons.

* Peptides A1, A4, and A5 were not detected, presumably due to their small size or unusual protein properties.

* Numbers in parentheses correspond to mass values obtained by subtracting the experimentally determined mass from the predicted mass and reflect mass increases.

* Peptide A6/7 corresponds to the uncleaved dipeptide of A6 plus A7.

* Relative amounts of modified or unmodified peptides A2 and A6/7 were determined by a method similar to that described in Table II.

modified fragment (A2M\(a\)) was detected with a mass increase of 162 daltons (Table III). As observed for peptide C3M\(a\), the A2M\(a\) peptide most likely contained two acyl-glycine ethyl ester adducts among the five glutamic acid residues located between Leu-25 and Ser-39.

In order to identify the precise sites of adduction, modified and unmodified peptide C3 were purified to apparent homogeneity by DEAE-cellulose chromatography (data not shown). Amino acid sequence analysis unambiguously identified peptide C3 from CNBr-treated Ugi forms I–IV. Examination of the amino acid PTH-derivatives detected in sequencing cycles for the C3 peptide of Ugi form II revealed peaks of a unique PTH-derivatized amino acid appearing at cycles 4 and 7 (Fig. 11), thus identifying Glu-28 and Glu-31 as sites of modification. The unique amino acid derivative peaks concordantly appeared, corresponding to decreases in Glu-28 and Glu-31, and similar peaks were not detected at other cycles containing Glu or Asp residues. Almost equal amounts of the novel amino acid derivative were detected at cycles 4 and 7 for both forms II and III. Interestingly, an additional unique PTH-derivatized amino acid was detected for Glu-28 and Glu-31 in Ugi form IV, suggesting two types of adducts (Fig. 9). Taken together, these results identify Glu-28 and Glu-31 as two major sites of EDC/GEE modification.

**DISCUSSION**

We have demonstrated that EDC/GEE modification of Ugi protein resulted in the selective adduction of specific glutamic acid residues that inactivated Ugi activity. Ugi form I represented unmodified inhibitor protein, since it did not exhibit any neutralization of charge, increase in mass, or reduction in inhibitor activity. Ugi form II contained a single adduct per peptide. We assumed that the low abundance of this modified species and interference by minor mass peaks in the C4 region obscured detection following CNBr cleavage of Ugi form II. Ugi form III digested with endoproteinase Asp-N also produced peptides A2M\(a\) and A6/7M\(a\); however, an additional
**Uracil-DNA Glycosylase Inhibitor Protein**

**FIG. 11.** Amino acid sequence determination of the Ugi form II C3 peptide. A histogram shows the relative amount of a unique PTH-derivative detected during each cycle of Edman degradation of Ugi form II C3 peptide. This novel derivative appeared with a retention time of 15.54 ± 0.05 min between standards identified as Arg (~15.17 min) and Tyr (~16.13 min). The amino acid sequence determined for the C3 peptide that overlaps with the A2 peptide is indicated.

Amino Acid: Leu Pro Glu Val Glu Val Ile Gly Asn Lys Pro Glu Ser

The results presented in this investigation provide evidence that Glu-28 and Glu-31, located in the α2-helix of Ugi, play an important role in promoting stable Ung–Ugi complex formation. Involvement of these two residues was implied, since 82% of Ugi form II adducts were detected on peptide A6/7 (residues 61–84) but represented only ~18% of the total Ugi form II adducts. Ugi form III was shown to contain two acyl-glycine ethyl ester adducts, and 65–70% of these protein molecules had adducts at both Glu-28 and Glu-31 sites. The remaining molecules of form III Ugi apparently possessed a single adduct on both peptide A2 and A6/7, since the amount of peptide A2M1 (~25%) nearly equaled that of A6/7M1 (37%). The type, location, and distribution of each adduct on Ugi forms IV and V became more difficult to exactly define due to the heterogeneity of modification. We suspect that the second PTH-derivative associated with form IV peptide C3 represents an N-acrylurea adduct formed by an internal rearrangement of acylisoura (36).

The results presented in this investigation provide evidence that Glu-28 and Glu-31, located in the α2-helix of Ugi, play an important role in promoting stable Ung–Ugi complex formation. Involvement of these two residues was implied, since 82% of Ugi form II and III contained at least one adduction in the α2-helix sequence. Thus, little room is left to explain the 42 and 67% reduction in Ugi activity by another modified site. However, we cannot discount the possible involvement of minor adducts in influencing the stability of some complexes. We speculate that both Glu-28 and Glu-31 contribute to the formation of a stable Ung–Ugi complex. This proposal is reinforced by recent x-ray crystallographic studies of the human and herpes simplex virus type-1 uracil-DNA glycosylase-Ugi complexes (4, 22). Both structures reveal that residues of the Ugi α2-helix and adjoining β1 strand dominate the interface with the enzyme and provide sites of interaction between the two proteins. The hydrophobic face of the α2-helix in conjunction with the β-sheet forms a hydrophobic pocket that surrounds the conserved Leu-272 active site loop of human uracil-DNA glycosylase (4). This pocket appears to be stabilized by Ugi Glu-28 and Glu-31 contacts with conserved residues that are shared between human and *E. coli* Ung (3, 4). These interactions involve the Ugi Glu-28 carboxylate forming a pair of hydrogen bonds with the Ser-247 (Ser-166 of *E. coli* Ung) backbone amide and side chain O-γ (4). In addition, Glu-28 O-ε1 also forms water-mediated hydrogen bonds with the human His-268 backbone amide and Ser-273 O-γ, corresponding to *E. coli* Ung His-187 and Ser-192, respectively (4). By the same token, Ugi Glu-31 forms a salt bridge with Arg-276 of human uracil-DNA glycosylase (Arg-195 of *E. coli* Ung). Thus, EDC/GEE modification of either Glu-28 or Glu-31 might be expected to disrupt important protein/protein interactions and destabilize the complex as observed in this investigation.

Several lines of evidence suggest that the interactions between Ugi Glu-28 and Glu-31 with Ung influence the formation of a tight Ung–Ugi complex without dramatically impeding the association of the initial precomplex. Steady-state kinetic experiments that monitored the binding of Ugi forms I–V to F-Ung demonstrated that neither modified nor unmodified Ugi freely dissociated from Ung once the final complex was achieved. However, the structure of these complexes differed since binding of Ugi forms II–V resulted in reduced levels of maximal fluorescence quench. We observed from competition binding experiments that unmodified Ugi preferably formed a complex over the modified protein species. Furthermore, unmodified Ugi was observed to replace modified Ugi (forms II–V) from a preformed complex. These properties of Ugi forms II–V were revealing, since unmodified Ugi maintains an irreversible Ung–Ugi complex (19). Thus, we conclude that Ugi forms II–V were defective in achieving an irreversible association with Ung. Observations indicating that poly(U) promoted dissociation of modified Ugi from complex and that preformed Ung–Ugi (form II–V) complexes displayed uracil-DNA glycosylase activity also support this interpretation.

The results provide new insight concerning the structural and functional relationship of the Ugi α2-helix in complex formation. Previously, stopped-flow kinetic experiments indicated that the Ung–Ugi association involved a two-step mechanism composed of a docking and locking reaction. The docking reaction was shown to involve a rapid pre-equilibrium in which Ung and Ugi associated to form a reversible precomplex (19). It is likely that the transition between the docking and locking steps involves an isomerization reaction. This could be achieved by a conformational change in Ung, Ugi, or both proteins. A comparison of the crystal structures of free human and herpes simplex virus type-1 uracil-DNA glycosylase with each respective enzyme in complex with Ung indicates that only minor changes occur within the enzyme tertiary structure upon complex formation (21, 37). A similar comparison between the NMR tertiary structure of unbound Ugi with the crystal structure of Ung complexed with either the human or herpes simplex virus type-1 enzyme reveals that significant structural changes occur in Ung (4, 22, 24). Direct evidence that Ugi undergoes a conformational change upon binding to Ung was provided by NMR results (24). The heteronuclear multiple quantum correlation spectroscopy spectrum of free [15N]Ugi compared with that of the Ung[15N]Ugi complex clearly indicates that many Ung residues undergo a conformational change as a consequence of forming the locked complex. One of the most dramatic rearrangements appears to involve the location of the Ugi α2-helix. In solution, the α2-helix is extended away from
the β-sheet core of Ugi (24), whereas in complex it is folded across the β-sheet, allowing Glu-28 and Glu-31 to interface with the DNA-binding cleft of uracil-DNA glycosylase where Ugi Glu-28 occupies the same spatial coordinates of a DNA phosphate group in UngDNA molecular models (4, 22). We speculate that movement of the α2-helix, at least in part, transforms the reversible Ung-Ugi precomplex to an essentially irreversible locked state. This interpretation is consistent with the observation that Ugi forms II–V are capable of successfully conducting the docking but not the locking interaction.

The proposed conformational change in the Ugi α2-helix provides an attractive explanation that unifies the biochemical, genetic, and structural data. However, several issues remain to be elucidated regarding the role of Glu-28, Glu-31, and other residues in the Ung/Ugi interaction. First, what is the relative involvement of Glu-28 and Glu-31 in facilitating the locking reaction? Second, does charge neutralization of these carboxyl groups alone bring about inactivation of Ugi, or does EDC/GEE modification sterically hinder α2-helix positioning? Third, what other amino acids play a role in the docking and locking interaction? Site-directed mutagenesis and additional protein structural studies should reveal the function of various Ugi amino acids in the Ung-Ugi complex.

Acknowledgments—We thank Lilo Barofsky for performing mass spectrometry through the Environmental Health Sciences Center, Mass Spectrometry Core Unit, and Barbara Robbins for conducting the amino acid sequence analysis through the Center for Gene Research and Biotechnology, Oregon State University. We also acknowledge Amy Lundquist for assistance in preparing the figures.

REFERENCES

1. Mosbaugh, D. W., and Bennett, S. E. (1994) Prog. Nucleic Acid Res. 48, 315–370.
2. Lindahl, T., Ljunquist, S., Siegert, W., Nyberg, B., and Sperens, B. (1977) J. Biol. Chem. 252, 3296–3294.
3. Olsen, L. C., Aasland, R., Wittwer, C. U., Krokan, H. E., and Helland, D. E. (1989) EMBO J. 8, 3121–3125.
4. 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.
5. Roberts, R. J. (1995) Cell 82, 9–12.
6. Higley, M., and Lloyd, R. S. (1993) Mutat. Res. 294, 109–116.
7. Bennett, S. E., Sanderson, R. J., and Mosbaugh, D. W. (1995) Biochemistry 34, 6109–6119.
8. Dianov, G., Price, A., and Lindahl, T. (1992) Mol. Cell. Biol. 12, 1605–1612.
9. Dianov, G., and Lindahl, T. (1994) Curr. Biol. 4, 1069–1076.
10. Singhal, R. K., Prasad, R., and Wilson, S. H. (1995) J. Biol. Chem. 270, 949–957.
11. Takahashi, I., and Marmur, J. (1963) Nature 197, 794–795.
12. Wang, Z., and Mosbaugh, D. W. (1989) J. Biol. Chem. 264, 1163–1171.
13. Savva, R., and Pearl, L. H. (1995) Proteins Struct. Func. Genet. 22, 287–289.
14. Friedberg, E. C., Ganesan, A. K., and Minton, K. (1975) J. Virol. 16, 315–321.
15. Wang, Z., Smith, D. G., and Mosbaugh, D. W. (1991) Gene (Amst.) 99, 31–37.
16. Bennett, S. E., and Mosbaugh, D. W. (1992) J. Biol. Chem. 267, 22522–22521.
17. Karran, P., Cone, R., and Friedberg, E. C. (1981) Biochemistry 20, 6992–6996.
18. Caradonna, S., Ladner, R., Hansbury, M., Kocziuk, M., Lynch, F., and Muller, S. (1996) Exp. Cell Res. 222, 345–359.
19. Bennett, S. E., Schimerlik, M. I., and Mosbaugh, D. W. (1993) J. Biol. Chem. 268, 26879–26885.
20. Bennett, S. E., Jensen, O. N., Barofsky, D. F., and Mosbaugh, D. W. (1994) J. Biol. Chem. 269, 21870–21879.
21. Mol, C. D., Arvai, A. S., Slupphaug, G., Kavli, B., Aasland, R., Krokan, H. E., and Tainer, J. A. (1995) Cell 80, 869–878.
22. Savva, R., and Pearl, L. (1995) Nat. Struct. Biol. 2, 752–757.
23. Balasubramanian, S., Beger, R. D., Bennett, S. E., Mosbaugh, D. W., and Bolton, P. H. (1995) J. Biol. Chem. 270, 296–303.
24. Beger, R. D., Balasubramanian, S., Bennett, S. E., Mosbaugh, D. W., and Bolton, P. H. (1995) J. Biol. Chem. 270, 16840–16847.
25. Demena, J. D., and Mosbaugh, D. W. (1985) Biochemistry 24, 7320–7328.
26. Mosbaugh, D. W., and Linn, S. (1983) J. Biol. Chem. 258, 108–118.
27. Laemmli, U. K. (1970) Nature 227, 680–685.
28. Reisner, A. H. (1984) Methods Enzymol. 104, 439–441.
29. Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404–427.
30. Haugland, R. P. (1992) in Handbook of Fluorescent Probes and Research Chemicals (Larson, K. D., ed) 5th Ed., p. 22, Molecular Probes, Inc., Eugene, OR.
31. Jensen, O. N., Barofsky, D. F., Young, M. C., von Hippel, P. H., Swenson, S., and Seifried, S. E. (1993) Rapid Commun. Mass Spectrom. 7, 456–501.
32. Carraway, K. L., and Koshland, D. E. (1972) Methods Enzymol. 25, 614–623.
33. Geren, L. M., O’Brien, P., Stonehuerner, J., and Millett, F. (1984) J. Biol. Chem. 259, 2155–2160.
34. Buechler, J. A., and Taylor, S. S. (1990) Biochemistry 29, 1937–1943.
35. Medina, M., Pieleato, M. L., Mendez, E., and Gomez-Morena, C. (1992) Eur. J. Biochem. 203, 373–379.
36. Timkovich, R. (1977) Anal. Biochem. 79, 135–143.
37. Savva, R., McAuley-Hecht, K., Brown, T., and Pearl, L. (1995) Nature 373, 487–493.
38. Lee, T. D., and Vemuri, S. (1989) Proceedings of the 37th American Society of Mass Spectrometry Conference, May 21–26, Miami Beach, FL.
39. Varshney, U., Hutcheon, T., and van de Sande, J. H. (1988) J. Biol. Chem. 263, 7776–7784.