Kinetics of the Uracil-DNA Glycosylase/Inhibitor Protein Association

Ung INTERACTION WITH Ugi, NUCLEIC ACIDS, AND URACIL COMPOUNDS*

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The bacteriophage PBS2 uracil-DNA glycosylase inhibitor (Ugi) inactivates Escherichia coli uracil-DNA glycosylase (Ung) by forming an Ung–Ugi protein complex with 1:1 stoichiometry. Stability of the Ung–Ugi complex was demonstrated by the inability of free Ugi to exchange with Ugi bound in preformed complex. Ung was reacted with fluorescein 5-isothiocyanate to produce fluorescent-Ung (F-Ung), which retained full uracil-DNA glycosylase activity and susceptibility to Ung inactivation. Addition of Ugi to F-Ung under steady-state conditions resulted in saturable (15%) fluorescence quenching at a F-Ung–Ugi ratio of 1:1.4. Dissociation constants determined for the F-Ung interaction with M13 DNA, uracil-containing DNA, and poly(U) equaled 600, 220, and 190 μM, respectively. While F-Ung associated with nucleic acid polymers was able to bind Ugi efficiently, F-Ung bound in the F-Ung–Ugi complex could no longer effectively bind nucleic acid. Stopped-flow kinetic analysis suggested the F-Ung/Ugi association was described by a two-step mechanism. The first step entailed a rapid pre-equilibrium distinguished by the dissociation constant $K_d = 1.5 \mu M$. The second step led irreversibly to the formation of the final complex and was characterized by the rate constant $k = 195 s^{-1}$. We infer Ugi inactivates Ung through the formation of an exceptionally stable protein–protein complex.

Uracil residues in DNA are promptly removed by the uracil-excision repair pathway (1, 2). Such repair is initiated by the action of uracil-DNA glycosylase, which cleaves the N-glycosylic bond between uracil and the deoxyribose phosphate backbone (3). The coordinated action of uracil-DNA glycosylase with apurinic/apyrimidinic endonucleases, deoxyribophosphodiesterase, DNA polymerases, and DNA ligase facilitates DNA repair (4–7).

Uracil-DNA glycosylase has been purified from diverse biological sources, ranging from bacteria to human cells (3, 8–12). Significant amino acid similarity (40–50% identical residues) has been reported between uracil-DNA glycosylase from Escherichia coli and that of yeast, herpes virus, and human placenta (13, 14). E. coli uracil-DNA glycosylase (Ung) is a monomeric protein that has a deduced molecular weight of 25,664 (15), an isoelectric point of 6.6 (16), a 2-fold substrate specificity for uracil residues in single-stranded over double-stranded DNA, and no cofactor requirement (3). The enzyme is inhibited by both reaction products: uracil and AP-containing DNA. Uracil acts as a noncompetitive inhibitor with $K_i$ values of ~2 mM and 0.12 mM reported for single-stranded and double-stranded DNA substrates (3). The other reaction product, the AP site, acts as a competitive inhibitor at concentrations 2–3 orders of magnitude lower than uracil (17). However, the AP site analogues deoxyribose and deoxyribose 5'-phosphate are not inhibitory at $\leq 1$ mM concentrations (18).

Ung is also inhibited by the bacteriophage PBS2 uracil-DNA glycosylase inhibitor protein Ugi (19, 20). The bacteriophage ugi gene has been cloned, sequenced, and overexpressed in E. coli (20–22). Ugi is a monomer ($M_r = 9477$), heat-stable, acidic ($pI = 4.2$) protein (16). In vitro studies indicated that Ugi specifically inactivates Ung and has no inhibitory effect on any of the other DNA metabolizing enzymes tested (21, 23). However, Ugi has been shown to inactivate uracil-DNA glycosylases isolated from dissimilar biological sources (21). Recently, Ugi was shown to inactivate Ung by forming a stable protein complex with 1:1 stoichiometry (16). The Ung–Ugi complex, stable under physiological conditions, can be reversed by treatment with SDS or 8 M urea at 70 °C. Ung and Ugi recovered from the binary complex exhibit no change in electrophoretic mobility, apparent molecular weight, or specific activity relative to controls (16). Therefore, the probable mode of inhibitor action involves direct and exclusive binding to Ung.

EXPERIMENTAL PROCEDURES

Materials—Fluorescein 5-isothiocyanate (FITC isomer I) was purchased from Molecular Probes and isopropyl-1-thio-β-4-galactopyranoside came from Life Technologies, Inc. Restriction enzymes (Aul, BamHI, DraI, EcoRI, HindIII, Hpal, Smal) came from Life Technologies, Inc. [3H]Leucine and [3SS]methionine were obtained from DuPont NEN; [3H]dUTP was from Amersham Corp. [γ-32P]ATP was from New England Nuclear Corp.

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E. coli strains JM105 (Δlac-proAB), thi, rpl, endA, sbcB15, hsdR4/F, traD36, proAB, lacIΔZAM15) were provided by W. Ream (Oregon State University); C3368 (dut-1, ung-1, thi-1, relA1/pCJ105 (Cm)) was a gift from T. A. Kunkel (National Institute of Environmental Health Sciences) as was the bacteriophage M13mp2. Plasmid pK223-3 was obtained from Pharmacia LKB Biotechnology Inc. pW2Tac1 (22) and pBD396 (25) were constructed as previously described.

The abbreviations used are: Ung, uracil-DNA glycosylase; Tris, Tris(hydroxymethyl)aminomethane; IPTG, isopropyl-1-thio-β-4-galactopyranoside; AP, apurinic/apyrimidinic; FITC, fluorescein 5-isothiocyanate; F-Ung, fluorescein 5-isothiocyanate-conjugated uracil-DNA glycosylase.
Uracil-DNA Glycosylase-Inhibitor Complex

Construction of Ung Overexpression Plasmid—The pBD396 Hpal-HpaII restriction fragment containing the E. coli ung gene was inserted into the Smal site of the expression vector pKK223-3. Subsequently, the Drel-HindIII fragment of the recombinant plasmid was subjected to limited AluI digestion, and the AluI-HindIII fragment encompassing the 31.5-kb region was isolated and pre-incident with Smal and HindIII restriction endonucleases. In the resulting plasmid (pSB1051), ung expression was under control of the IPTG-inducible tac promoter.

Purification of [3H]Uracil-DNA Glycosylase and Inhibitor Protein—E. coli JM105 cells transformed with pSB1051 were grown at 37 °C until an 8-liter volume of M9 medium supplemented with 100 mg/ml thiamine, 0.01% ampicillin, and 40 mg each of the 20 common amino acids. Upon reaching a density of 4 x 10^8 cells/ml, the cells were pelleted by centrifugation at 3500 x g for 15 min in a GSA (Sorval) rotor and resuspended in 1.5 liters of M9 supplemented medium without leucine. Growth was then resumed until the cell density reached 6 x 10^8 cells/ml, at which time 1.5 mcg of [3H]leucine (158 Ci/mmol) were added to the medium. When the cell density reached 7.5 x 10^8 cells/ml, IPTG was added to 1 mM, which induced [3H]Ung overproduction. After 3 h at 37 °C, the E. coli cells were harvested by centrifugation as indicated above. [3H]Ung purification was carried out as previously described (16) except that the concentration step of the Ung. Ugi complex was resolved from its component proteins using a DEAE-cellulose column (0.2 cm x 5 cm) equilibrated at 4 °C in 0.05 M potassium phosphate (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, and 5% (w/v) glycerol. Protein bands were in approximately 1:1 stoichiometry. The existence of Ung and/or Ugi preparation might have been inactive. To examine this possibility, [3H]Ung was co-eluted with [35S]Ugi as complex; no free [3H]Ung was detected in the wash fractions. Thus, an excess of inhibitor labels represented the Ung-Ugi complex as the radioactivities detected in the wash fractions. Consequently, [3H]Ung in cell extracts of IPTG-induced E. coli JM105/pSB1051 was found to be ~10-fold higher than that of uninduced cells and 910-fold higher than untransformed bacteria. We estimate from a densitometric scan (Fig. 1B, lane 2) that Ung accounted for 13% of the total soluble protein in the IPTG-induced E. coli JM105/pSB1051 extract. Following overexpression of ung in [3H]leucine-supplemented M9 minimal medium, [leucine-3H]uracil-DNA glycosylase ([3H]Ung) was purified to apparent homogeneity (Fig. 1B, lane 6). [3H]Ung (Fraction V) was used in all of the experiments described below unless otherwise indicated.

Resolution of the Ung-Ugi Complex from Component Proteins by DEAE-Cellulose Chromatography.—We have previously demonstrated that the uracil-DNA glycosylase inhibitor (Ugi) protein forms a stoichiometric complex with Ung (16, 21). In order to investigate the stability of the Ung-Ugi complex and to determine if Ung in preformed complex could exchange with free Ung, we developed a chromatographic technique for resolving Ung-Ugi from its component proteins. Samples containing either [3H]Ung or [35S]Ugi were applied to DEAE-cellulose columns and eluted with two steps of equilibration buffer: one containing 150 mM NaCl and the other 250 mM NaCl. Under these conditions, [3H]Ung eluted at 495 nm; fluorescence emission was monitored using a Corning 3-72 cut-off filter. Data were fitted by means of Marquard's algorithm in a computer program modified from Duggleby (29).

Fluorescent Measurements—Steady-state fluorescent measurements were conducted at 23 °C using a photon-counting spectrophotofluorometer (SLM 8000) equipped with a 450-watt xenon arc lamp and a thermostatted mini-cell (4 mm) with constant stirring. Excitation and emission wavelengths were 496 and 520 nm, respectively, both full widths at half maximum were set at 8 nm. Stop-flow experiments were carried out at 25 °C using a Dione D137 stopped-flow photometer (dead time = 2.3 ms) equipped with a 75-watt xenon lamp and interfaced to a model 206 Nicolet digital storage oscilloscope. Excitation occurred at 495 nm; fluorescence emission was monitored using a Corning 3-72 cut-off filter. Data were fitted by means of Marquardt's algorithm in a computer program modified from Duggleby (29).

RESULTS

Subcloning and Overexpression of the Uracil-DNA Glycosylase Gene—An expression vector pSB1051 (Fig. 1A) was constructed to overproduce E. coli uracil-DNA glycosylase and facilitate purification of the enzyme. In this construct the ung promoter was removed and the IPTG-inducible tac promoter inserted upstream of the ung gene. After transforming E. coli JM105 with pSB1051, we found that Ung was overproduced in response to addition of 1 mM IPTG (Fig. 1B, lanes 1 and 2). Specific activity of Ung in cell extracts of IPTG-induced E. coli JM105/pSB1051 was found to be ~10-fold higher than that of uninduced cells and 910-fold higher than untransformed bacteria. We estimate from a densitometric scan (Fig. 1B, lane 2) that Ung accounted for 13% of the total soluble protein in the IPTG-induced E. coli JM105/pSB1051 extract. Following overexpression of ung in [3H]leucine-supplemented M9 minimal medium, [leucine-3H]uracil-DNA glycosylase ([3H]Ung) was purified to apparent homogeneity (Fig. 1B, lane 6). [3H]Ung (Fraction V) was used in all of the experiments described below unless otherwise indicated.

Resolution of the Ung-Ugi Complex from Component Proteins by DEAE-Cellulose Chromatography.—We have previously demonstrated that the uracil-DNA glycosylase inhibitor (Ugi) protein forms a stoichiometric complex with Ung (16, 21). In order to investigate the stability of the Ung-Ugi complex and to determine if Ung in preformed complex could exchange with free Ung, we developed a chromatographic technique for resolving Ung-Ugi from its component proteins. Samples containing either [3H]Ung or [35S]Ugi were applied to DEAE-cellulose columns and eluted with two steps of equilibration buffer: one containing 150 mM NaCl and the other 250 mM NaCl. Under these conditions, [3H]Ung eluted in the wash fractions (Fig. 2A). In contrast, [35S]Ugi was found to elute after the 250 mM NaCl step (Fig. 2B). When [3H]Ung was incubated with an equal molar amount of [35S]Ugi and applied to the column, a major peak containing [3H] and [35S] labels co-eluted during the 150 mM NaCl step (Fig. 2C). We concluded that the peak containing both [3H] and [35S] labels represented the Ung-Ugi complex as the radioactivities were in approximately 1:1 stoichiometry. The existence of minor peaks containing either [3H]Ung or [35S]Ugi suggested that some fraction of the Ung and/or Ugi preparation might have been inactive. To examine this possibility, [3H]Ung was combined with a 3-fold molar excess of [35S]Ugi and subjected to chromatography (Fig. 2D). This time all of the [3H]Ung co-eluted with [35S]Ugi as complex; no free [3H]Ung was detected in the wash fractions. Thus, an excess of inhibitor drove the enzyme completely into complex. In a similar experiment, excess [3H]Ung was added to [35S]Ugi; however, only 82% of the [35S] label was converted to the complex (data not shown). Taken together, these results argue that whereas all the glycosylase was capable of complex formation, approximately 18% of the inhibitor preparation may have been inactive.

To determine if Ugi in complex with Ung could exchange
A. [Diagram of Uracil-DNA Glycosylase-Inhibitor Complex]

B. [MW (x10³) 1 2 3 4 5 6 Lane]

FIG. 1. Overproduction and purification of [³H]Ung from pSB1051. A, the overexpression vector SB1051 was constructed by insertion of the E. coli ung gene-containing AluI-HindIII fragment of pBD396 into pKK223-3, as described under “Experimental Procedures.” The predicted translational start site of the gene is located -37 base pairs from the 3’ end of the tac promoter. B, [³H]Ung was purified from 1.5 liters of IPTG-induced E. coli JM105/pSB1051 as described under “Experimental Procedures.” The cell-free extract of uninduced cells (lane 1) and purification fractions I-V (lanes 2-6), containing 25, 25, 25, 10, 5, and 2.5 µg of protein, respectively, were analyzed on a 12.5% polyacrylamide gel containing SDS. The proteins were visualized after staining with Coomassie Brilliant Blue G-250. The molecular size standards for bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, and trypsin inhibitor are indicated by arrows from top to bottom, respectively. The location of the tracking dye (TD) and Ung are shown by arrows. Specific activity was determined as described previously (16).

with free Ugi, [³H]Ung was incubated with a 3-fold molar excess of nonradioactive Ugi and DEAE-cellulose chromatography was performed to resolve the constitutive components (Fig. 3A). As expected, all of the [³H]Ung formed complex and eluted at 150 mM NaCl. To test for Ugi exchange, we incubated the [³H]Ung-Ugi complex, then added either a 3-fold or 30-fold molar excess of [³⁵S]Ugi. After allowing time for exchange to occur, the possible protein components ([³H] Ung-Ugi, [³H]Ung-[³⁵S]Ugi, [³⁵S]Ugi) were resolved by chromatography (Fig. 3, B and C). Under both conditions, we did not observe any significant incorporation of [³⁵S]Ugi into complex. Thus, we conclude that significant exchange did not occur and that the [³H]Ung-Ugi complex was exceptionally stable.

Fluorescein 5-Isothiocyanate Labeling and Properties of F-Ung—To further investigate the properties, kinetics, and mechanism of the Ung/Ugi interaction, the [³H]Ung preparation was fluorescently labeled. The standard modification reaction (pH 10) resulted in an average of 3 fluorescein molecules bound/ enzyme molecule (Table I). Under these conditions we observed no significant change in the specific activity of the labeled enzyme or in the ability of Ugi to inhibit
TABLE I

Effect of various reaction conditions on fluorescein conjugation and specific activity of uracil-DNA glycosylase

| pH | [FITC]| [Ung] | FITC specific activity | Ugi inhibition |
|----|-------|-------|-----------------------|----------------|
| 9.0 | 2.5 | 1.2 | 54 (120) |
| 9.5 | 2.7 | 1.3 | 59 (120) |
| 10.0 | 3.0 | 1.0 | 31 (97) |
| 10.5 | 4.2 | 0.4 | 27 (46) |

* After removing the unreacted FITC, the concentration of fluorescein-conjugated Ung to Ugi was determined by absorbance spectrophotometry using the extinction coefficient: ε 495 nm = 7.8 × 10^5 M^−1 cm^−1. To determine the [Ung] concentration in radioactivity of 80-μl samples was measured by liquid scintillation spectrometry using 5 ml of Formula 989 as fluor. The specific radioactivity of [3H]Ung was determined to be 12.2 cpm/μmol. The degree of fluorescein substitution is the molar ratio of the concentrations of bound-fluorescein to enzyme.

Uracil-DNA glycosylase activity of the fluorescein-conjugated enzyme preparation was determined using the standard assay described under “Experimental Procedures.” Protein concentrations were determined from the specific radioactivity of [3H]Ung as described above.

The uracil-DNA glycosylase inhibitor assay was performed as described under “Experimental Procedures.” A stock solution of Ugi (66 μg/ml) was diluted 1:10,000, 1:50,000, and 1:100,000, and 25-μl aliquots were introduced into reaction mixtures containing the following amounts of FITC-treated or untreated uracil-DNA glycosylase: pH 9.0, 0.1 unit; pH 9.5, 0.07 unit; pH 10.0, 0.08 unit; pH 10.5, 0.08 unit, and pH 10.5, 0.08 unit, respectively. The amount of Ugi inhibition is expressed as the percent to which the enzyme activity was diminished by Ugi relative to the control lacking Ugi. The amount of inhibition was normalized to that observed at a 1:50,000 dilution of Ugi. Numbers in parentheses represent the percent inhibition observed in the FITC-treated enzyme versus the untreated preparations.

Ump, UDP, and UTP or the corresponding deoxy-derivatives had no effect on F-Ung fluorescence or enzyme activity (data not shown).

To determine whether fluorescence quenching was quantitative, we titrated F-Ung with Ugi and monitored the relative fluorescence (Fig. 5A). Subsequent to Ugi addition, F-Ung emitted a steady fluorescent signal, which decreased in direct proportion to the amount of added Ugi. The response was saturated at high Ugi concentrations; the maximum decrease in fluorescence intensity observed was 15%. We conclude that changes in F-Ung fluorescent intensity reflected Ugi binding and that the F-Ung-Ugi complex was rapidly formed (<30 s).

When the change in fluorescent intensity was plotted against Ugi concentration (expressed as a molar ratio to F-Ung concentration), a sharp break in the titration curve suggested a stoichiometry of 1:1 (F-Ung-Ugi) (Fig. 5B). This result was consistent with the previously determined stoichiometry of 1:1 (16), particularly in light of the observation that ~18% of the inhibitor preparation may have been functionally inactive (Fig. 3C). Similar titration experiments were conducted with M13 DNA, uracil-containing DNA, and poly(U) (data not shown). Since the fluorescent intensities of free and nucleic acid-saturated F-Ung were known, the fraction of bound enzyme (Fb) at a particular nucleic acid concentration could be calculated from the formula Fb = (Fr - Fb)/(Fr - Fc), where Fr represents the fluorescence of F-Ung after an addition of nucleic acid, Fc the fluorescence of free F-Ung, and Fb fluorescence at saturation. The Kd values calculated for M13 DNA, M13 uracil-DNA and poly(U) were 600, 220, and 190 μM, respectively.

Effect of Ugi on Ung Binding to Nucleic Acids—The influence of Ugi on F-Ung binding to M13 uracil-DNA was examined to determine whether the intensity of the fluorescence response was additive or exclusive. When F-Ung was combined with a saturating amount of uracil-DNA (1500 μM), fluorescent intensity was characteristically quenched by ~6% (Fig. 6A, △RF). The addition of a 10-fold excess of Ugi caused a further decrease in fluorescent intensity of ~9% (Fig. 6A, △RF), resulting in a total decrease in intensity of 15% (△RF). In the converse experiment, F-Ung was first combined with 10-fold excess of Ugi and the fluorescence signal was again quenched 15% (Fig. 6B, △RF). In this case, when uracil-DNA was added, no further decrease in fluorescent intensity was observed (Fig. 6B), leading us to postulate that the Ugi interaction with F-Ung precludes subsequent enzyme binding to uracil-DNA. Analogous sets of experiments were...
with buffer dependence; no change in fluorescence intensity was observed.
Flow methods. The kinetics of the interaction were examined using stopped-flow methods. The interaction with F-Ung was completed within 30 min.

Ung (control) 24,240
+Ugi 20,530 15.3 94.6 98
+1 mM uracil 24,300 <0.1 104 20
+1 mM deoxyribose 5'-phosphate 24,170 0.3 90.0 5
+500 µg/ml M13 DNA 22,660 6.5 70.0 26
+300 µg/ml M13 U-DNA 22,760 6.1 53.9 43
+500 µg/ml poly(U) 22,910 5.5 63.4 33
+500 µg/ml poly(A) 24,190 0.2 90.8 4

Also conducted using M13 DNA and poly(U) with very similar results (data not shown). We interpret these findings to indicate that the Ung-Ugi complex cannot effectively bind nucleic acid.

**Stopped-flow Kinetic Analysis of Ugi Binding to Ung—**

Because steady-state experiments indicated that the Ugi interaction with F-Ung was completed within 30 s after mixing, the kinetics of the interaction were examined using stopped-flow methods. As a control, F-Ung (100 nM) was combined with buffer A and the fluorescent signal examined for time-depence; no change in fluorescence intensity was observed (Fig. 7A). However, when F-Ung was mixed with excess Ugi (600 nM), two widely separated kinetic phases of interaction were observed: a “fast” ($t_{1/2} \sim 10$ ms) phase and a “slow” ($t_{1/2} \sim 1.7$ s) phase (Fig. 7, B and C). The decrease in F-Ung fluorescence in the fast phase appeared to follow a single exponential decay: $F(t) = A_0 + A e^{-k_{obs}t}$ (fast). Slow phase data were also fitted to a single exponential, providing that data from the first 100 ms representing the fast phase were omitted from the analysis. In order to explore the dependence of $k_{obs}$ on Ugi concentration, F-Ung was reacted with a range of Ugi concentrations, and the fast and slow binding phases were analyzed separately (Fig. 8, A and B). The curve for the fast phase was hyperbolic, indicative of the presence of a pre-equilibrium step prior to final complex formation. In contrast, the dependence of the slow phase $k_{obs}$ on Ugi concentration was linear, implicative of the absence of an equilibrium step prior to final complex formation. In view of the fact that the majority of the fluorescent decrease was attributable to the fast phase, we surmised that the slow phase represented a parallel reaction involving a subclass of F-Ung. The simplest mechanism consistent with the fast phase data is given in Scheme 1, where $E$ represents F-Ung and $I$ the inhibitor Ugi.

$$E + I = K E I$$

**SCHEME 1**

First, there is a rapid pre-equilibrium step, characterized by the dissociation constant $K$, in which Ung and Ugi associate to form a pre-complex $E-I$. The final form of the complex,
F. Ung, Ugi interaction was shown to be noncovalent in vitro to be noncovalent (16). The results show that Ugi bound in a preformed complex was not capable of exchange with free Ugi in solution. Addition of either a 3- or 30-fold molar excess of [35S]Ugi to preformed [3H]Ung-Ugi complex did not eventuate detectable incorporation of [35S]Ugi into complex. Since Ung did not appear to contribute more significantly to the reaction as fluorescence modification of the enzyme increased. This correlation suggests that the slow phase may have been produced as an artifact of protein modification.

**DISCUSSION**

We have determined a kinetic mechanism for the Ugi inactivation of Ung. We find that the complex is essentially irreversible under physiological conditions, although the Ung/Ugi interaction has been shown to be noncovalent (16). The results show that Ugi bound in a preformed complex was not capable of exchange with free Ugi in solution. Addition of either a 3- or 30-fold molar excess of [35S]Ugi to preformed [3H]Ung-Ugi complex did not eventuate detectable incorporation of [35S]Ugi into complex. Since Ung completely quenched fluorescence observed in the slow phase, we formed the Ung-fluorescein conjugate under various pH conditions and FITC concentrations (Table I). When the labeling reaction was conducted at pH 9–10, the specific activity and Ugi inhibition of F-Ung were relatively unchanged before and after the conjugation reaction. Under these conditions, the ratio of FITC bound per Ung increased slightly from 2.5 to 3.0. In contrast, the Ung preparation that was reacted at pH 10.5 with a 100-fold excess of FITC possessed only 31% of the glycosylase activity and 46% of the Ugi inhibition relative to the untreated control. This F-Ung preparation appeared to contain additional FITC modifications, as the fluorescein:Ung ratio was 4.2:1. Thus, FITC modification of Ung above a 3:1 ratio seemed to alter F-Ung, causing loss of activity. Three F-Ung preparations (pH 9, 10, and 10.5) were mixed with 600 nM Ugi, and stopped-flow kinetic measurements were determined in a series of slow (20-s duration) kinetic traces. The initial amplitudes were aligned to a common ordinate value and the traces superimposed, and the first second of each time course was examined (Fig. 9). The observed rate constants of the fast and slow phase of the pH 9 and 10 preparations were similar; however, the fast phase rate constant of the pH 10.5 preparation was 2.5-fold lower. The contribution of the fast phase to the total decrease in fluorescence was different for each preparation. Furthermore, the contribution of the fast phase of the total change in fluorescence decreased with increased FITC labeling of Ung. While the slow phase appeared to contribute more significantly to the reaction as fluorescence modification of the enzyme increased. This correlation suggests that the slow phase may have been produced as an artifact of protein modification.
inactivates the host-uracil-DNA glycosylase within 4 min post-infection (19), the formation of a high affinity, stable complex is not unexpected. Indeed, the biological role of Ugi, preservation of the PBS2 phase uracil-DNA genome through inactivation of the host uracil-DNA glycosylase, would be best served if the Ung-Ugi complex were essentially irreversible in vivo.

Fluorescein 5-isothiocyanate has been shown to preferentially conjugate with lysine residues and exhibit excitation and emission spectra with peaks at wavelengths of 495 and 520 nm, respectively (30). FITC modification of Ung produced the anticipated spectrum, suggesting lysine modification. Enzyme modified with an average of 2.5-3 fluorescein residues displayed normal specific activity and inhibition by Ugi. When the extent of modification reached an average of 4.2 fluoresceins, both enzyme activity and sensitivity to Ugi were reduced to 50% that of the unmodified control. These results suggest that a lysine residue critical to enzyme function may have been rendered inactive or, alternatively, that the conjugation of additional lysines distorts the active conformation of Ung.

The effect of Ugi and uracil compounds on the steady-state fluorescence and enzyme activity of F-Ung revealed a correspondence between binding (quenching of F-Ung fluorescence) and inhibition, with uracil being the one important exception. The addition of Ugi in 10-fold excess to F-Ung caused the largest fluorescence quench (~15%) and inhibition (>98%), whereas the addition of nucleic acid polymers in 10-fold excess of nucleotides resulted in less quenching (~5%) and inhibition (26-43%). It is possible that Ung adopts different conformations, as reflected by FITC quenching, when it binds to nucleic acids as opposed to Ugi. Alternatively, Ugi binding may occlude a significant portion of the solvent-accessible surface of the enzyme, resulting in a larger fluorescence quench. Various uracil-containing nucleosides as well as deoxyribose 5-phosphate neither quenched F-Ung fluorescence nor caused inhibition. These results are in accordance with the observation that the uracil moiety must reside in DNA to be substrate, albeit the length of DNA may as short as 2-3 nucleotides (31). When uracil was added to F-Ung, no quenching of fluorescence was observed; however, enzyme activity was inhibited by 81%. The lack of fluorescence quenching by uracil is subject to several interpretations. For example, the uracil binding site might lie outside the vicinity of a fluorophore; if so, uracil binding would not be reported. In addition, uracil binding might result in a conformational change that is not reported by any fluorophore. If the uracil binding site were synonymous with the catalytic site, a significant separation may exist between the DNA binding domain and the catalytic site. The catalytic site may reside interior to the DNA binding site and remain inaccessible to FITC. The ratio of uracil to Ung in a typical inhibition reaction is 10:1. If the Ung-uracil binding interaction were transient and if free uracil molecules were able to penetrate the DNA binding and/or catalytic site, it is possible that a decrease in fluorescence might not be detected. It is noteworthy that the other reaction product, AP site in DNA, is inhibitory at concentrations 100-fold lower than uracil (17).

The results of stopped-flow kinetic studies of the F-Ung/Ugi interaction indicated that fluorescent quenching proceeded by two widely separated phases: a very fast phase followed by a slower phase. However, subsequent analysis of the dependence of the observed rate constants on Ung concentration disclosed that the slow phase was linear (Fig. 8). The hyperbolic dependence of the fast phase rate constant on Ung concentration denoted the presence of a pre-equilibrium step during the fast phase of Ung-Ugi interaction; however, the linearity of the slow phase with respect to Ung concentration was indicative of the absence of a pre-equilibrium step prior to formation of final complex. Since the fast phase occurs prior to the slow phase, the effect of an initial equilibrium step would be evidenced in the slow phase (i.e. the slow phase curve should also have been hyperbolic). It seemed reasonable that the interaction between Ung and Ugi might include a preliminary “docking” step, through which optimal alignment of the two proteins could be achieved. This step could be considered to be reversible if correct alignment was not obtained and would produce the observed hyperbolic curve. We surmised that the slow phase might represent a parallel reaction carried out by a subset of the F-Ung population, possibly that portion of the preparation which was FITC-modified at critical lysine residues. To test this hypothesis, we compared the kinetics of three F-Ung preparations of varying dye:protein stoichiometry. The contribution of the fast phase reaction to the total change in fluorescence decreased with increased FITC labeling of Ung, while that of the slow phase increased correspondingly. Moreover, preparation of F-Ung with the highest dye:protein stoichiometry (4:2:1) was considerably less active, both enzymatically and specifically by the initial equilibrium when Ugi. This may suggest that Ung binds at or near the DNA binding site of the enzyme and that extensive FITC modification either sterically hindered Ugi binding or altered the Ung conformation to impair or block Ung interaction. Since Ugi is a nonglobular, negatively charged (PI = 4.2) protein (16), it may resemble the phosphodiester backbone of DNA. It remains to be determined whether Ugi acts as an alternate substrate of high affinity or inactivates Ung by distorting the Ung-DNA binding site.

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