

5-Formyluracil-Induced Perturbations of DNA Function[†]Daniel K. Rogstad,[‡] Jiyoung Heo,[§] Nagarajan Vaidehi,[§] William A. Goddard III,[§] Artur Burdzy,[‡] and Lawrence C. Sowers^{*,‡}*Department of Biochemistry and Microbiology, Loma Linda University School of Medicine, Loma Linda, California 92350, and Materials and Process Simulations Center, Beckman Institute (139-74), California Institute of Technology, Pasadena, California 91125**Received November 26, 2003; Revised Manuscript Received March 8, 2004*

ABSTRACT: Oxidation of the thymine methyl group can generate 5-formyluracil (FoU), which is known to be both mutagenic and chemically unstable in DNA. Synthetic oligonucleotides containing FoU at defined sites have been prepared to investigate potential mechanisms by which FoU might perturb DNA function. The half-life of the glycosidic bond of an FoU residue in single-stranded DNA under physiological conditions of temperature and pH is estimated to be approximately 148 days, orders of magnitude shorter than the parent pyrimidine, thymine. This reduced stability of FoU residues in DNA is attributed to the inductive properties of the 5-formyl substituent. Oxidative modification of the thymine methyl group could also inhibit association with sequence-specific DNA-binding proteins. Alternatively, the 5-formyl substituent of FoU could cross-link nonspecifically with protein amino groups. Transcription factor AP-1 is known to make specific contacts with thymine methyl groups of DNA in its recognition sequence. Substitution of T by FoU is shown to inhibit AP-1 (c-Jun homodimer) binding with a $\Delta\Delta G$ of approximately 0.6 kcal/mol. No evidence of cross-link formation is observed with either AP-1 or polylysine. Molecular modeling studies on the FoU-containing oligonucleotide sequence corresponding to the duplex used in the experimental studies demonstrate that the 5-formyl substituent of an FoU residue paired with adenine lies in the plane of the pyrimidine base and is well protected from solvent on one face and only partially accessible on the other. The results of this study suggest that although FoU residues in DNA are considerably more labile than thymine, they are likely to be present long enough to miscode as well as interfere with DNA–protein interactions.

The DNA of all living organisms is constantly damaged by endogenous oxidation and hydrolysis as well as by exogenous genotoxic chemical and physical agents (1–3). It is estimated that the total number of such lesions may exceed 10 000 per cell per day under normal metabolic conditions (2–3). Among these damage products are those that arise from the oxidation of the thymine methyl group, including 5-hydroxymethyluracil (1, 3) and 5-formyluracil (Figure 1; 4–7).

Unlike HmU,¹ which does not miscode (8), 5-formyluracil (FoU) residues in DNA are known to miscode with relatively high frequency, generating primarily transition mutations (7, 9–14). The strong miscoding potential of FoU has been attributed to the strong electron-withdrawing 5-formyl substituent that increases substantially the acidity of the N³ proton (9). Additionally, the electron-withdrawing 5-sub-

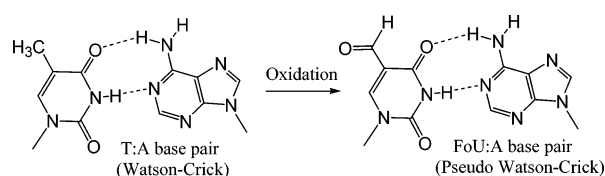


FIGURE 1: Oxidation of thymine (T) in a T·A base pair to 5-formyluracil (FoU), resulting in a pseudo-Watson–Crick FoU·A base pair.

stituent destabilizes the N-glycosidic bond, rendering FoU-containing oligonucleotides highly susceptible to hydrolysis (5). However, no studies to date have measured the half-life of the glycosidic bond of FoU in DNA. Although FoU has been shown to be mutagenic, it is possible that under physiological conditions FoU could be spontaneously hydrolyzed from the DNA and repaired before it has the chance to miscode. Alternatively, if the error is not recognized and repaired, the resulting abasic site could generate a mutation different from that normally caused by the FoU base itself.

In addition to altering the electronic properties of the pyrimidine base, the oxidation of the thymine methyl group to the 5-formyl substituent would be expected to interfere with sequence-specific DNA–protein interactions. Recently, we demonstrated that the replacement of T by HmU did substantially reduce binding of the AP-1 transcription factor (15). Although it has yet to be shown, the 5-formyl substituent of FoU could additionally form potentially lethal

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¹ Abbreviations: FoU, 5-formyluracil; FodU, 5-formyl-2'-deoxyuridine; U, uracil; HmU, 5-hydroxymethyluracil; RP-HPLC, reversed-phase high-performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry; UDG, uracil DNA glycosylase; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; TBE, Tris–borate–EDTA buffer; Tris, tris(hydroxymethyl)aminomethane.

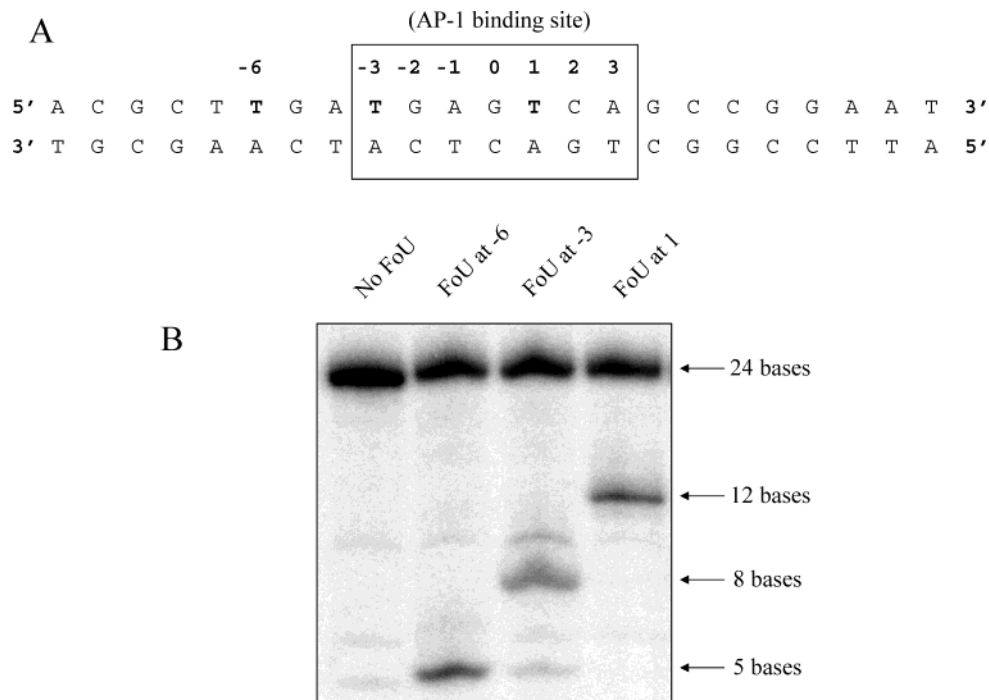


FIGURE 2: (A) Oligonucleotide 23-mer sequence used in our studies, containing the AP-1 consensus binding sequence. (B) Identification of FoU in oligonucleotides with sequence in panel A, containing FoU in place of thymine in the positions indicated. Alkaline conditions (0.10 M piperidine, 90 °C, 30 min) were used to selectively cleave oligonucleotides at positions containing FoU, and oligonucleotides were resolved on a 20% denaturing polyacrylamide gel.

covalent cross-links with an amino group of DNA-binding proteins, resulting in locking the binding instead of blocking binding (16–19).

In this paper, synthetic oligonucleotides have been constructed containing FoU residues at defined sites. These oligonucleotides have been used to probe the lability of the glycosidic bond of FoU-containing residues in oligonucleotides and to determine if the presence of the FoU residue in duplex oligonucleotides interferes with sequence-specific DNA–protein interactions or instead results in cross-link formation. It is observed that FoU residues are substantially more labile in DNA than the parent residue thymine and that the enhanced rate of glycosidic bond hydrolysis can be attributed to the inductive properties of the 5-formyl substituent. In the system examined here, the replacement of T by FoU does inhibit the binding of the AP-1 transcription factor, and the magnitude of the interference is similar to that observed for HmU and U substitutions. No evidence is observed for the formation of DNA–protein cross-links with either the AP-1 transcription factor or polylysine. The sequence studied experimentally was also examined with molecular mechanics methods. The modeling studies suggest that the steric accessibility of the 5-formyl substituent of FoU in duplex DNA is substantially reduced. The results of this study indicate that although the FoU lesion in DNA is orders of magnitude more labile than the parent thymine, it is sufficiently stable to survive long enough to miscode during DNA replication, and if not repaired, it could generate point mutations and interfere with sequence-specific DNA–protein interactions.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis, Purification, and Characterization. The 5-formyl-2'-deoxyuridine phosphoramidites and

5-formyluracil-containing oligonucleotides were synthesized by a modification of the method of Berthod et al. (20). Unprotected FodU (21) was converted to the 5'-dimethyltrityl, 3'-cyanoethylphosphoramidite by established methods (22). Oligonucleotides were synthesized with phosphoramidites designed for mild deprotection by use of methanolic potassium carbonate (23). Oligonucleotides were purified by RP-HPLC and further purified by denaturing polyacrylamide gel electrophoresis. The sequence studied here is 23 bases long (23-mer) and contains the AP-1 transcription factor consensus binding site (Figure 2A). One strand of each oligonucleotide duplex pair was 5'-³²P end-labeled by T4 polynucleotide kinase (New England Biolabs) with [γ -³²P]-ATP (ICN). Labeled oligonucleotides were purified from excess free ³²P label after reactions by using P6 Micro Bio-Spin chromatographic columns (Bio-Rad). Presence of FoU bases in oligonucleotides was confirmed by hydrolyzing unlabeled oligonucleotides at 140 °C in formic acid, followed by heating at 140 °C in acetonitrile and bis(trimethyl silyl)-trifluoroacetamide for silylation and then analyzing by GC/MS. To confirm the correct placement of FoU in oligonucleotides, 3.0 pmol of labeled single-stranded oligonucleotides were incubated with 10% (v/v) piperidine (20 μ L) at 90 °C for 30 min, as FoU in DNA is known to be piperidine-labile (10). Mixtures were dried under vacuum and residual piperidine was coevaporated twice with 20 μ L of water. DNA was resuspended in 20 μ L of 98% formamide loading buffer, heat-denatured at 90 °C for 5 min, chilled on ice for 1 min, and loaded onto a 20% denaturing polyacrylamide gel (19:1 acrylamide:bisacrylamide) run with 1 \times TBE buffer. Gels were prewarmed at 400 V for about 30 min before 10 μ L of each sample was loaded and run at 500 V for at least 70 min. Wet gels were wrapped in plastic and exposed on a phosphor screen, and the radioactivity was visualized by use

of a PhosphorImager (Molecular Dynamics). To obtain duplexes, labeled oligonucleotides were heated with a 2-fold molar excess of their unlabeled complementary oligonucleotides in 10 mM Tris-HCl (pH 7.5) and 1.0 mM EDTA at 60–65 °C for 5–10 min and allowed to cool slowly to room temperature to promote annealing. As a control, oligonucleotides annealed at 60 °C were run on a nondenaturing gel to confirm duplex formation.

FoU Hydrolysis Measurement and Calculations. To determine the rate of hydrolysis of the glycosidic bond of FoU in DNA, 2.0 pmol of an oligonucleotide duplex containing FoU in one position on the ³²P-labeled strand was heated in 10 mM Tris-HCl and 1.0 mM EDTA (pH of buffer was measured to be 7.36) for 15 min–8 h at either 75, 85, or 95 °C. Reactions (20 μL) were heated in microcentrifuge tubes and subsequently allowed to cool at room temperature for at least 30 min to reanneal to their complementary strands. Following hydrolysis and cooling, oligonucleotides were frozen at –80 °C until the reactions for that set of time points were finished. Then all were treated with 2.5 units of human AP endonuclease (Trevigen) at 37 °C for 1 h under the following conditions: 10 mM HEPES–KOH (pH 6.5), 100 mM KCl, 10 mM MgCl₂, 5% glycerol (v/v), and 0.1 mg/mL BSA. Reactions were stopped by adding an equal volume of 98% formamide loading buffer, and competitor oligonucleotides were added in at least 270× molar excess. Mixtures were heat-denatured at 95 °C for 3 min, cooled quickly on ice, and loaded onto a 20% denaturing polyacrylamide gel. Gels were prepared, run, and analyzed as described above. Quantitation of gel bands was done with ImageQuant 5.0 (Molecular Dynamics). The sequence of the ³²P-labeled strand used in these hydrolysis experiments was 5′-d(ACGCTTGATGAG[FoU]CAGCCGGAAT)-3′. The corresponding double-stranded oligonucleotide containing all T (no FoU) has an estimated melting temperature of 63.7 °C in 0.1 M NaCl and duplex concentration of 0.1 μM (24–26).

Hydrolysis of the glycosidic bond of FoU in 5′-³²P-labeled FoU-containing oligonucleotides and cleavage of the phosphate backbone by AP-endonuclease gives rise to labeled oligonucleotides of shorter length, according to the position of the FoU base. Integration of the rate equation for the concentration of FoU-containing oligonucleotides as a function of the rate constant and time (*t*) is given in eq 1, where [FoU-oligo]₀ is equal to the initial concentration of ³²P-labeled uncleaved FoU-containing oligonucleotide:

$$[\text{FoU-oligo}]_t = [\text{FoU-oligo}]_0 e^{-kt} \quad (1)$$

Rate constants are first-order in oligonucleotide and were determined by measuring the ratio of uncleaved and cleaved labeled oligonucleotide as a function of time. Each rate constant was determined by plotting the natural logarithm of [FoU-oligo]₀/[FoU-oligo]_{*t*} versus reaction time. Data were normalized to unheated minus controls, and linear least-squares regression (via Microsoft Excel 2000) was used to generate a straight line through the data points whose slope is equal to the rate constant, *k*. The corresponding half-life for each rate constant was calculated by solving eq 1 for *t* when the ratio [FoU-oligo]_{*t*}/[FoU-oligo]₀ = 0.5. The activation energy, *E*_{act}, was determined from the Arrhenius equation:

$$\ln k = \ln A - \frac{E_{\text{act}}}{RT} \quad (2)$$

which involves measuring the rate constants as a function of temperature. When the natural logarithm of the rate constants is plotted versus the inverse of the temperature (in kelvins), the slope of the line obtained is equal to $-E_{\text{act}}/R$, where *R* is the gas constant (27, 28).

Gel Mobility Shift Assay and Binding Calculations. Binding of the AP-1 transcription factor to end-labeled annealed oligonucleotides was assayed by incubating human recombinant AP-1 c-Jun (Promega) with the appropriate oligonucleotides and resolving the complexes on nondenaturing 13% polyacrylamide gels (19:1 acrylamide:bisacrylamide) in 1× TBE buffer. Procedures for the binding assays, gel mobility shift assays, and calculations are as reported in Rogstad et al. (15). The ΔΔ*G* calculations on FoU inhibition of AP-1 binding are comparisons of free energy changes between the unsubstituted (all T-containing) oligonucleotide duplex and the duplexes containing FoU. A positive ΔΔ*G* means decreased binding of the FoU-substituted oligonucleotide by AP-1 compared to the unsubstituted oligonucleotide, and a negative ΔΔ*G* means increased binding. Averages of four measurements were made for each duplex: two each at 1.1 μM and 2.2 μM AP-1 (c-Jun), according to eqs 3 and 4:

$$K_d = \sqrt{\frac{[\text{c-Jun}]_{\text{total}}^2 (1 - y)}{y}} \quad (3)$$

where [c-Jun]_{total} is the concentration of AP-1 (c-Jun monomer) added to the binding reactions, *y* = fraction bound oligonucleotide, and *K*_{*d*} is the dissociation constant, the concentration at which half of the oligonucleotides are bound by AP-1 (c-Jun), or *y* = 0.5:

$$\Delta\Delta G = -RT \ln \left[\frac{K_d(\text{control})}{K_d(\text{substitution})} \right] \quad (4)$$

where *R* = 1.987 × 10^{−3} kcal mol^{−1} K^{−1} and temperature *T* = 277 K (4 °C, the temperature of the ice–water bath in which experiments were performed).

Cross-Linking Assays. Both single- and double-stranded oligonucleotides containing FoU in one position (same sequence as that used for hydrolysis experiments, see above) were incubated with polylysine (poly-L-lysine hydrobromide, molecular mass ~ 10 kDa) and subsequently run on denaturing and nondenaturing polyacrylamide gels to observe possible cross-links between FoU and amines in the polypeptides. Mixtures were incubated for 2 h at either room temperature or 37 °C and included 4 μL of DNA (0.50 pmol), 1–2 μL of polylysine (0.33 ng/μL), and either water or 10 mM Tris-HCl (pH 7.5) and 1.0 mM EDTA (1× TE buffer) to a total volume of 15 μL. Reactions containing water were dried under vacuum, resuspended in 5 μL of water and 5 μL of 98% formamide loading buffer, and run on a denaturing 20% polyacrylamide gel. Glycerol and nonformamide loading buffer were added to reactions containing 1× TE buffer and run on a nondenaturing 13% polyacrylamide gel. Gel conditions and visualization have been described above.

Molecular Modeling. Quantum mechanical (QM) calculations were performed to calculate the energy difference and the energy barrier between the syn and anti conformations of the 5-formyl group of FoU. The torsional barrier of the 5-formyl group was computed for 5-formyl-1-methyluracil in gas phase with the MSCFF4.2 force field (29) and was compared with one obtained by QM calculation at the B3LYP/6-31G**++ level to verify the torsion barrier energy in the force field. Subsequently we calculated the energy difference and torsional barrier between the syn and anti conformations using the force field MSCFF (29) for a 9-mer double-stranded oligonucleotide. The partial atomic charges for FoU were obtained by fitting the electrostatic potential from the QM calculations. The force field parameters for FoU were assigned by use of the MSCFF (29) force field, which is based on the universal force field (30). Subsequently, we built a double-stranded 9-mer oligonucleotide, with sequence 5'-d(TGAG[FoU]CAGC)-3' in accordance with our 23-mer (Figure 2) with the program Polygraf. Eighteen sodium cations were added to neutralize the negative charge on the phosphate group. The sodium ions were placed initially at 2.25 Å from P on the O-P-O plane. The B-DNA duplex with the sodium ions was immersed in a periodic box of 267 water molecules (31). Water molecules having bad van der Waals contacts with the DNA were removed from the box. The potential energy of the water molecules and ions were first minimized by use of conjugate gradients with the DNA fixed. Subsequently all the atoms in the whole system were minimized to 0.1 kcal mol⁻¹ Å⁻¹ in force/atom. The conjugate gradient minimizations and all the molecular mechanics force field calculations in this paper have been performed with the Cerius2 program (32). The nonbond interactions were calculated by the Ewald summation method (33).

RESULTS

Identification of FoU in DNA. Following synthesis and purification of the oligonucleotides with and without FoU present, oligonucleotides containing FoU were 5'-³²P-labeled and treated with hot piperidine to test for correct placement of FoU in the DNA. These reactions were run on a denaturing polyacrylamide gel and are shown in Figure 2B.

Hydrolysis of FoU in Single-Stranded DNA. The stability of the glycosidic bond linking FoU to the DNA backbone in a single-stranded oligonucleotide containing FoU was tested as a function of time and temperature. A 5'-³²P-labeled oligonucleotide containing FoU at position 1 (Figure 2A) was heated at 75, 85, and 95 °C (pH ~7.4), and the lability of the glycosidic bond was measured by calculating the ratio of cleaved to uncleaved oligonucleotide on denaturing polyacrylamide gels. Five time points were used for each experiment (ranging from 15 min to 8 h) as well as a minus control (not heated, kept at room temperature until the following step), and each rate constant and half-life of the FoU base in DNA was determined at three different temperatures (75, 85, and 95 °C). The three rate constants obtained were used to calculate the activation energy for FoU as described under Experimental Procedures. The rate constants, each corresponding half-life, and the calculated activation energy for FoU are shown in Table 1. Plots of data are shown in Figure 3.

Table 1: Rate Constants, Half-Lives, and Activation Energy (E_{act}) for Hydrolysis of FoU in Single-Stranded DNA^a

temp (K)	rate constant k (s ⁻¹)	half-life $t_{1/2}$ (h)	E_{act} (kcal/mol)	R^2
348	6.98×10^{-6}	27.6	27.4	0.947
358	2.13×10^{-5}	9.04		0.955
368	6.02×10^{-5}	3.20		0.936
				1.00

^a Rate constants, k , were obtained by solving the following time-dependent equation for k : $[\text{FoU-oligo}]_t = [\text{FoU-oligo}]_0 e^{-kt}$, where $[\text{FoU-oligo}]_0$ = initial concentration of ³²P-labeled uncleaved FoU-containing oligonucleotide. The energy of activation (E_{act}) was calculated according to the Arrhenius equation: $\ln k = \ln A - E_{act}/RT$. See Experimental Procedures for oligonucleotide sequence used.

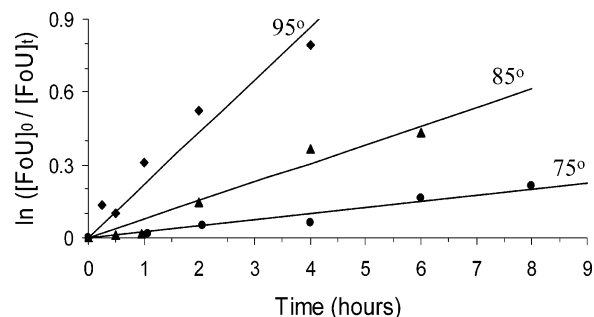


FIGURE 3: Kinetic data for the rate of FoU hydrolysis in single-stranded DNA for three different temperatures. Rate constants and corresponding half-lives as well as the activation energy are reported in Table 1.

Table 2: Substitutions Made at Thymine Residues Outside and Inside the AP-1 Binding Site^a

Duplex Number	Duplex Sequence	$\Delta\Delta G$ (kcal/mol)
1	5' -ACGCTTGA TGAGTCA GCCGGAAT -3' 3' -TGCCTTAA ACTCAGT CGGCCTTA -5'	
2	5' -ACGCTFGA TGAGTCA GCCGGAAT -3' 3' -TGCCTTAA ACTCAGT CGGCCTTA -5'	-0.04
3	5' -ACGCTTGA FGAGTCA GCCGGAAT -3' 3' -TGCCTTAA ACTCAGT CGGCCTTA -5'	0.32
4	5' -ACGCTTGA TGAGTCA GCCGGAAT -3' 3' -TGCCTTAA ACTCAGT CGGCCTTA -5'	0.58

^a $\Delta\Delta G$ is calculated by comparing the dissociation constants, K_d , for the binding of AP-1 (c-Jun) to the substituted and unsubstituted oligonucleotides, as discussed under Experimental Procedures (F = 5-formyluracil).

Inhibition of AP-1 Binding by FoU. Binding of AP-1 (c-Jun) to oligonucleotides with and without FoU were performed by the same methods as in our previous work on U- and HmU-containing oligonucleotides (15). Oligonucleotides shown in Table 2 were incubated with AP-1 (c-Jun homodimer), samples were run on non-denaturing polyacrylamide gels, bands were quantitated, and $\Delta\Delta G$ values were calculated as mentioned in the experimental procedures. Table 2 and Figure 4 both show the effects on AP-1 (c-Jun) binding by single T to FoU substitution both inside and outside the AP-1 binding site of the DNA. Substitution made outside of the binding site had no substantial effect on AP-1 (c-Jun) binding, whereas substitution inside the binding site resulted in a substantial reduction of binding as compared with the unsubstituted DNA sequence, with $\Delta\Delta G$ values between 0.32 and 0.58 kcal/mol.

Cross-Linking between FoU and Polypeptides. Both single- and double-stranded ³²P-labeled oligonucleotides

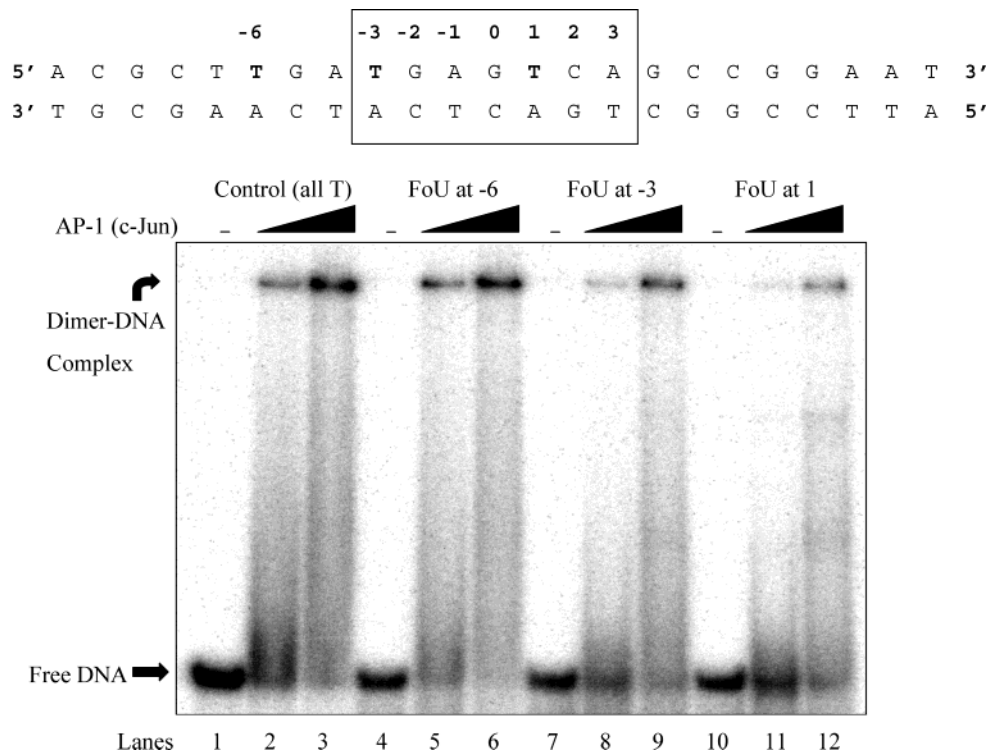


FIGURE 4: Inhibition of AP-1 (c-Jun) binding by replacement of T with FoU inside and outside the AP-1 binding region. The unsubstituted oligonucleotide and oligonucleotides containing a single FoU in different positions were compared by incubation with increasing amounts of AP-1 (c-Jun) and analyzed on a nondenaturing 13% polyacrylamide gel in a gel mobility shift assay.

Table 3: Energy Differences for Conformations of the 5-Formyl Group Relative to the Anti Conformation for 5-Formyl-1-methyluracil^a

conformation	QM ^b (gas)	QM ^c (aqueous)	MCFF4.2 (gas)
anti	0.0	0.0	0.0
perpendicular	11.8	9.6	10.9
syn	5.7	0.9	7.6

^a Energy differences are given in kilocalories per mole. ^b Zero-point energy-corrected. ^c Free energy at 300 K, by Poisson–Boltzmann continuum solvation method on Jaguar.

containing FoU in one position (oligonucleotide duplex 4 and its single-stranded counterpart; Table 2) were incubated with poly-L-lysine at physiological pH, at both room temperature and physiological temperature, and then run on both denaturing and nondenaturing polyacrylamide gels. Denaturing gels would allow visualization of stable covalent cross-links, whereas nondenaturing gels would allow visualization of cross-links that are more transient. No cross-links were observed under any of the conditions tested.

Molecular Modeling of FoU in Double-Stranded DNA. With 5-formyl-1-methyluracil as a model system, the 5-formyl group was rotated 90° to the anti or syn conformation and the energy difference between the perpendicular and anti conformations was calculated to be 10.9 kcal/mol in MCFF4.2 and 11.8 kcal/mol in QM as shown in Table 3. In both QM and force field calculations for the DNA base, the anti conformation has lower energy than the syn conformation. Here we can see that the MCFF4.2 results agree well with QM calculations (in gas phase).

Having validated this modeling approach with the 1-methylated free base, we then studied the sequence used for testing cross-linking in the experimental portion of this paper

(oligonucleotide duplex 4; Table 2) using the MSCFF force field. The minimized energy conformation of the DNA 9-mer is shown in Figure 5. To obtain the adiabatic rotational energy barrier of the 5-formyl group in double-stranded 9-mer oligonucleotides, in going from syn to anti conformation we varied the torsional angle connecting the -CHO group to the ring, by 10° at a step, and performed conjugate gradient minimization on the whole DNA system while the torsional angle was kept constrained on the set value. Two energy minima corresponding to the syn and anti conformations have been obtained. The chemical structures of FoU in the syn and anti conformations are shown in Figure 6, and a plot of the potential energy versus torsional angle is shown in Figure 7.

DISCUSSION

Oxidative damage to DNA results from a multitude of both endogenous and exogenous processes, forming thousands of lesions per cell per day (1–3). Among these is 5-formyluracil, a product of the oxidation of the thymine methyl group. It is reported that FoU is a major radiation-damaged DNA base (4–7), and several studies have reported that FoU residues in DNA are highly mutagenic (4, 7, 9–14). The conversion of thymine to FoU alters the chemical properties of the base, resulting in biochemical perturbations including miscoding during polymerase-directed DNA replication (10, 11, 13) and increased lability of the glycosidic bond (5, 10, 34), though the half-life of the bond in DNA has not been previously measured. Some studies have indicated the possibility for potential cross-links between FoU-containing DNA and proteins, though this has not yet been shown (16–19, 34, 35). Studies done with other bases modified at the 5-methyl group of thymine suggest that FoU could interfere with sequence-specific DNA–protein interactions (15, 36,

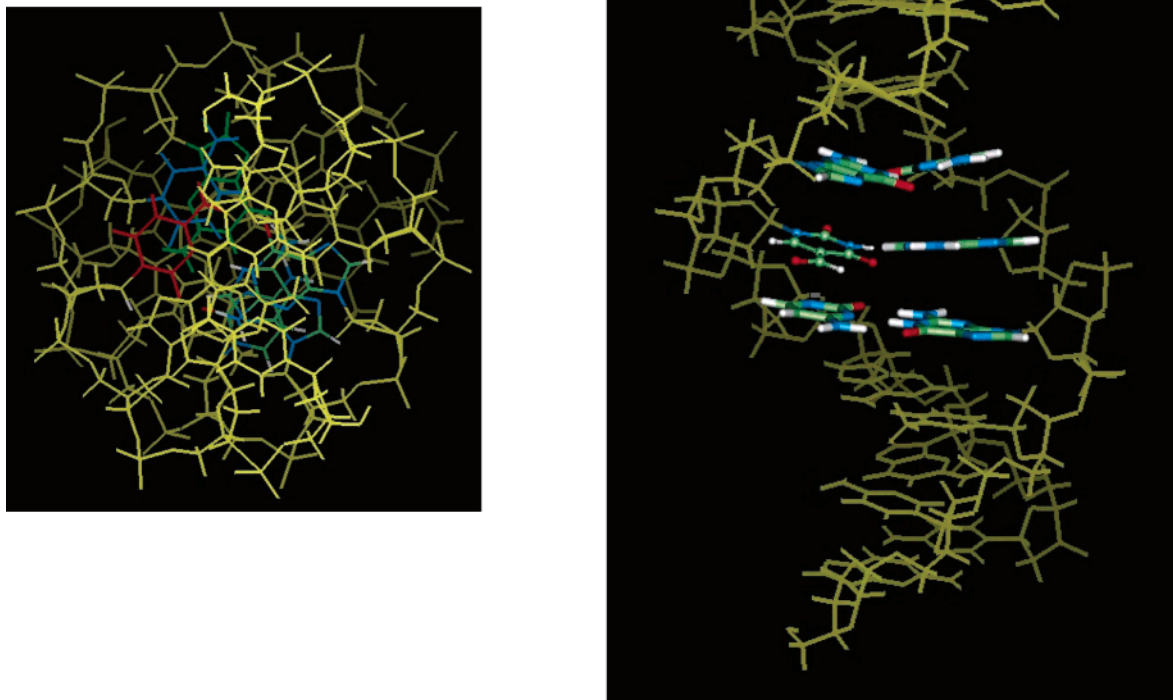


FIGURE 5: Model of 5-formyluracil (FoU) in the context of a minimized 9-mer double-stranded B-DNA helix with 5'-d(TGAG[FoU]-CAGC)-3'. (Right) Side view of the model. The FoU (center) is highlighted with adjacent bases. (Left) Top view of the model. The FoU is in blue, and the guanine and cytosine residues on either side are in green and red, respectively. The pictures were drawn with QUANTA98 software.

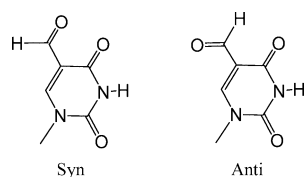


FIGURE 6: Chemical structures of FoU with the 5-formyl group in the syn and anti conformations.

37), and one study done with FoU confirms this, though the binding energy difference was not measured quantitatively (35). In this paper, we sought to quantify the chemical stability of FoU in synthetic oligonucleotides by measuring the half-life and activation energy of its glycosidic bond and to measure the impact of FoU on DNA–protein interactions.

Instability of the Glycosidic Bond. Previous studies have demonstrated that the glycosidic bond of 5-formyl-2'-deoxyuridine is substantially more labile than the parent thymidine. At neutral pH and 37 °C, FodU is converted to FoU with a half-life of 17 days (34). It is known that oligonucleotides bearing FoU residues may be cleaved at elevated temperatures, resulting from FoU release followed by hydrolysis of the phosphodiester backbone (5), but the half-life of the FoU–DNA glycosidic bond has never been measured.

In this study, an FoU residue was placed at a selected site in a synthetic oligonucleotide. The oligonucleotide was incubated in a buffered solution (pH ~7.4) at 75, 85 and 95 °C. The abasic site resulting from FoU release was cleaved with human AP-endonuclease, and the cleaved oligonucle-

otides were separated from the intact parent oligonucleotides by gel electrophoresis and quantified. The measured rates of FoU release from the synthetic oligonucleotides are shown in Table 1. Upon increasing the temperature from 75 to 95 °C, the half-life falls from 27.6 to 3.2 h. The hydrolysis rates we obtained are for single-stranded instead of double-stranded oligonucleotides, as the temperatures used are substantially higher than the melting temperature of our 23-mer double-stranded sequence. The duplex melting temperature is estimated at 63.7 °C (see Experimental Procedures) for the all T-containing double-stranded oligonucleotide and would be lower when an FoU residue is introduced (as in our hydrolysis experiments), which has been previously shown with FoU-containing oligonucleotide melting studies (38). The apparent activation energy is 27.4 kcal/mol, similar in magnitude to the activation energy for depurination (39) but lower than that measured for pyrimidine deoxynucleosides (40). On the basis of our data, we can estimate that the half-life for the cleavage of an FoU residue in a single-stranded oligonucleotide at 37 °C and pH 7.4 is approximately 148 days.

Deoxynucleosides are generally protected from hydrolysis when incorporated into oligonucleotides. Rate constants for hydrolysis generally fall by an order of magnitude when the hydrolysis rates for the free deoxynucleoside and the corresponding residue in a single-stranded oligonucleotide are compared (40). Thus, the half-life estimated for the FoU residue in DNA of 148 days is slightly less than that expected on the basis of the half-life of 17 days estimated for the free

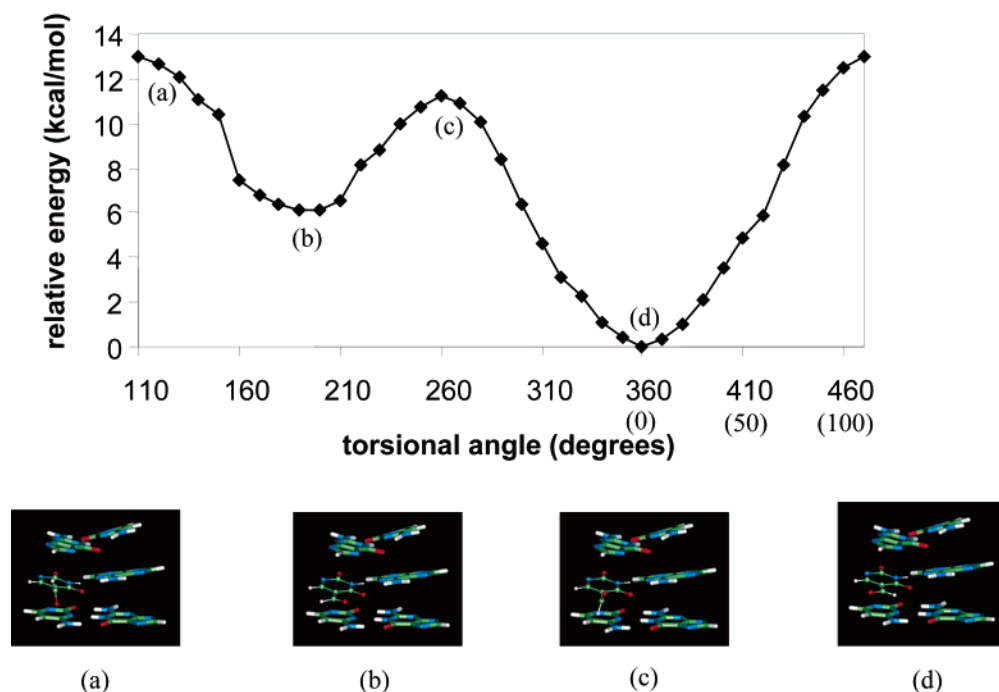


FIGURE 7: Energy barrier for the syn versus anti conformations of the 5-formyl group in 5-formyluracil within the context of double-stranded DNA: (a, c) perpendicular conformations; (b) syn conformation; (d) anti conformation.

deoxynucleoside (34). Conversion of single- to double-stranded DNA is known to reduce the rate of hydrolytic depurination by a factor of approximately 4 (39). Therefore, we estimate the half-life of an FoU residue in duplex DNA to be approximately 1.6 years.

Previously, Lindahl and Nyberg (39) estimated the rate constant for the depurination of double-stranded DNA (37 °C, pH 7.4) to be $k = 3 \times 10^{-11} \text{ s}^{-1}$. Extrapolating to single-stranded DNA, in which the rate would be increased by a factor of 4, the half-life for depurination is estimated to be approximately 200 years at 37 °C, pH 7.4. Subsequently, it was estimated that the rate of depyrimidination under similar conditions was 20-fold slower (40), resulting in a half-life of approximately 4000 years under physiological conditions of pH and temperature. By comparing the results of these previous studies (39, 40) with those reported here, we estimate that the oxidation of thymine to FoU would increase the rate of glycosidic bond cleavage by approximately 3–4 orders of magnitude.

The substantial increase in the rate of spontaneous, hydrolytic FoU release from DNA, relative to the parent thymine, can be attributed to the strongly electron-withdrawing 5-substituent. Shapiro and Kang (41) previously investigated the hydrolysis of the glycosidic bonds of thymidine, 2'-deoxyuridine, and 5-bromo-2'-deoxyuridine in solution. A linear relationship was observed between the logarithm of the rate constant for glycosidic bond cleavage and the pK_a of the N^1 proton of the free base, which can be described by

$$\log_{10} k = -0.993pK_a + 11.019 \quad (5)$$

The more electron-withdrawing the 5-substituent, the more acidic the N^1 proton and the greater the rate of glycosidic bond hydrolysis of the corresponding deoxynucleoside.

Previously, a linear Hammett relationship between the pK_a of the N^3 proton for a series of 5-substituted 2'-deoxyuridine

derivatives and the inductive property of the 5-substituent was established (9). Whereas the 5-methyl group is electron-donating to the pyrimidine ring, the 5-formyl substituent is strongly withdrawing. This led to the proposal that oxidation of the thymine methyl group to the formyl substituent of FoU would substantially increase miscoding during polymerase-directed DNA synthesis by increasing the proportion of the promutagenic, ionized base. Subsequent studies have provided substantial data in accord with this hypothesis (10, 11, 13).

A linear relationship was also observed between the inductive properties of the 5-substituent and the observed pK_a for the corresponding series of 5-substituted uracil derivatives. Three of the uracils, the 5-formyl, 5-carboxy, and 5-nitro derivatives, showed deviation from the Hammett relationship. Whereas the N^3 proton of thymine and the remaining uracil derivatives is the more acidic, it was proposed that the 5-substituted uracil derivatives bearing a carbonyl function on the 5-substituent, including 5-formyluracil, would facilitate ionization of the N^1 proton, as the resulting negative charge could be delocalized to the 5-substituent by resonance. Recently, the greater acidity of the N^1 proton of FoU has been confirmed by computational methods (42).

The pK_a values for the N^1 protons of thymine and FoU are 9.75 and 6.84, respectively (42). On the basis of the relationship established by Shapiro and Kang (eq 5) and the pK_a values for the N^1 position of thymine and 5-formyluracil, we would estimate the rates of glycosidic bond cleavage to be $k = 2.17 \times 10^{-7} \text{ s}^{-1}$ and $k = 1.69 \times 10^{-4} \text{ s}^{-1}$, respectively. Therefore, the oxidation of thymidine to FoU could be expected to increase the rate of glycosidic bond cleavage by a factor of ~ 1000 , on the basis of the change in the inductive properties of the 5-substituent. As this is similar in magnitude to the enhanced rate of hydrolytic loss of FoU from oligonucleotides discussed above, we conclude

that the substantial lability of FoU residues in DNA can be attributed to the reversal of the inductive property of the 5-substituent when the thymine methyl group is oxidized to the 5-formyl group. Interestingly, uracil ionized in the N¹-position has been postulated to be the intermediate in uracil glycosylase-mediated glycosidic bond cleavage (43). It is possible that DNA repair glycosylases could exploit chemical modification-induced perturbations in the electronic structure of damaged DNA bases as a mechanism for enhancing discrimination between damaged and undamaged bases in DNA (44).

Inhibition of AP-1 Binding by FoU in DNA. The FoU residue in an FoU·A base pair in DNA has been demonstrated by X-ray crystallography to assume a pseudo-Watson–Crick configuration (45). The 5-formyl substituent protrudes into the major groove of a B-form DNA duplex. The thymine 5-methyl group is known to be an important recognition element for the binding of sequence-specific DNA binding proteins (15, 35–37). Recently (15), it was predicted and confirmed experimentally that the oxidation of the thymine methyl group to the 5-hydroxymethyl substituent would interfere with the binding of the AP-1 transcription factor (c-Jun homodimer). A similar prediction was made for the substitution with the 5-formyl substituent. Furthermore, qualitative binding experiments done with FoU·A base pairs inside the binding region for the NF- κ B transcription factor suggest that FoU could affect binding of a protein to its DNA recognition sequence (35). Using our AP-1 system, we wanted to quantitatively test the effect of FoU on transcription factor binding, as well as to see if the FoU residue would block binding (such as seen with HmU) or lock binding (through protein–DNA cross-links). Oligonucleotides containing a single FoU residue at defined sites were incubated with AP-1 (c-Jun) and run on polyacrylamide gels in a gel mobility shift assay. Inhibition of binding was observed similar to that caused by HmU, resulting in a $\Delta\Delta G$ of as much as ~ 0.6 kcal/mol compared to the unsubstituted, all thymine-containing oligonucleotides. Under our conditions, it is clear that AP-1 binding to DNA is being blocked by FoU instead of locked into place through cross-linking, as the all T-containing DNA duplex binds with higher affinity than either duplex containing FoU within the AP-1 binding site (Figure 4).

Cross-Links between FoU and Amino Groups. Cross-links with FoU have been observed for the free deoxynucleoside [cross-linked to cysteine and related derivatives (34) and to a lysine derivative (35)] and 5-formyluridine 5'-triphosphate binding to RNA polymerase (16). FoU residues in single-stranded oligonucleotides have also recently been shown to cross-link covalently with peptides from the RecA protein (19), and it is possible that small organic molecules could cross-link with FoU residues in double-stranded oligonucleotides (18). However, although efforts have been underway to obtain cross-links between FoU in double-stranded DNA and proteins (35), such as the transcription factor NF- κ B, which contains a lysine residue very close to a thymine base in its recognition sequence (46, 47), this type of cross-linking has not yet been observed. As cross-links did not form between FoU-containing DNA and AP-1 under our non-denaturing polyacrylamide conditions, we were interested in testing to see if FoU in the context of double-stranded DNA would form cross-links with polypeptides containing amine

groups, such as polylysine. In this study, both single- and double-stranded oligonucleotides containing FoU were incubated with polylysine at physiological pH and temperature and no cross-links were observed when the oligonucleotides were examined under either denaturing or non-denaturing gel electrophoresis conditions. These observations suggest that the aldehyde group of FoU is protected from interacting with protein amino groups when incorporated into an oligonucleotide.

Examination of an FoU-Containing Oligonucleotide by Computational Methods. To better understand the apparent protection of the 5-formyl group of FoU from cross-linking while in double-stranded DNA, computations were performed to examine the orientation of the formyl group (Figures 5–7).

The computational model of the FoU-containing oligonucleotide duplex predicts that the formation of a base pair between FoU and adenine in pseudo-Watson–Crick geometry is favored, as shown in Figure 5. The rotational barrier for the 5-formyl substituent is high, preventing free rotation around this bond. Two energy minima corresponding to the syn and anti conformations have been calculated. A recent calculation from our group predicts that the anti conformation is preferred for the N¹-methylated FoU base (48), and this preference for anti is maintained in the duplex oligonucleotide structure containing FoU examined in this study. An amine group or other nucleophile attacking the 5-formyl substituent could approach perpendicular to the plane of the carbonyl group. Because the conformation of this formyl group is restricted and will be found in the plane of the pyrimidine ring, nucleophilic attack must also occur perpendicular to the plane of the FoU residue. In the duplex structure examined here, this position of attack is strongly eclipsed by the purine residue in the 5' direction. The opposing face of the FoU residue is more accessible to solvent. However, the amine groups in the side chains of lysine are mostly protonated at physiological pH and could form salt bridges with the negatively charged phosphates in the DNA duplex. These electronic interactions would slow the nucleophilic attack so that the kinetic energy would not be sufficient to condense with the 5-formyl group.

CONCLUSIONS

The hydrolytic stability of an FoU residue in a single-stranded oligonucleotide has been measured. The glycosidic bond of an FoU residue in DNA is about 3 orders of magnitude more labile than that of the parent pyrimidine, thymine (this study and refs 40 and 41), and is attributable to the electron-withdrawing 5-formyl substituent. The stability of FoU is enhanced upon incorporation into a single-stranded oligonucleotide, where it has a half-life of approximately 148 days, and presumably somewhat longer in double-stranded DNA. If not repaired by base excision repair, the FoU residue could survive long enough to miscode or to perturb DNA–protein interactions, particularly with sequence-specific DNA-binding proteins interacting with the thymine methyl group. The 5-formyl substituent of an FoU residue in duplex DNA appears to be well protected from condensation with peptides. Therefore, FoU-induced DNA–protein cross-links are unlikely to contribute substantially to the potential negative consequences of FoU formation in DNA.

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