NMR Evidence for an Unusually Low N1 pK_a for Uracil Bound to Uracil DNA Glycosylase: Implications for Catalysis

Alexander C. Drohat and James T. Stivers*

Center for Advanced Research in Biotechnology of the University of Maryland Biotechnology Institute and the National Institute for Standards and Technology 9600 Gudelsky Drive, Rockville, Maryland 20850

Received September 8, 1999
Revised Manuscript Received November 18, 1999

The first step of the DNA base-excision repair pathway is the removal of the damaged base by cleavage of the *N*-glycosidic bond through the action of a DNA glycosylase.[1] A key mechanistic question in these reactions is how the enzyme activates the expulsion of the leaving base. Solution and enzymatic studies have shown that purine bases may be activated by protonation or alkylation at N1, N3, or N7.[2] However, activation of a pyrimidine base by a similar mechanism involving protonation at O2 or O4 is energetically untenable due to the low pK_a (~ -3.4) of these carbonyl oxygens.[3] Thus, it is not obvious how pyrimidine specific DNA glycosylases activate the leaving group base.

A useful system to study these questions in detail is the relatively small and structurally well-characterized enzyme, uracil DNA glycosylase (UDG).[4] This enzyme uses base-flipping[5] and a simple hydrolytic mechanism to cleave the glycosidic bond of deoxyuridine in duplex and single strand DNA, giving the products uracil and abasic DNA (Figure 1).[6] We have recently reported NMR and kinetic evidence which indicates that UDG activates the uracil-leaving group by providing a short hydrogen bond from a neutral His187 to the developing negative charge on uracil O2 in the transition state (Figure 1).[6] Recent ^{1}H NMR experiments in our lab have revealed a highly deshielded resonance (δ = 15.6 ppm) in the ternary product complex at neutral pH that has been unambiguously assigned to this hydrogen bond.[7] This type of catalysis, involving a neutral imidazole and an enolate-like oxygen, is similar to that proposed for several enzymes that catalyze proton abstraction from a carbon atom alpha to a carbonyl group,[8] and becomes energetically more favorable as the difference in pK_a between the enolate and imidazole is decreased.[9] We show here, using a novel heteronuclear NMR approach, that the bound uracil base is anionic and in the N1−O2 imidate form at pH 7.5. These findings support our previously proposed mechanism that requires significant charge resonance to uracil O2,[6] and are inconsistent with recent proposals suggesting that a cationic histidine is involved in catalysis[10] and that the negative charge resonates predominantly to uracil O4.[11]

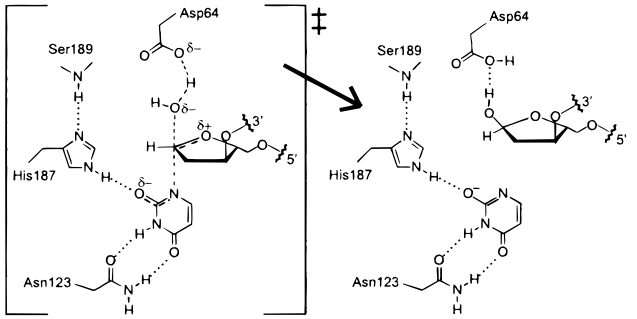

Figure 1. The proposed mechanism of *N*-glycosidic bond hydrolysis by UDG. Concerted electrophilic and general base catalysis by His187 and Asp64, respectively, is indicated.[6] An oxycarbenium ion transition state for the UDG reaction is shown on the basis of recent kinetic isotope effect measurements.[19] The interactions are based on the crystal structures of free and uracil-bound *Escherichia coli* UDG, and human UDG bound to the products abasic DNA and uracil.[4] NMR studies have established that His187 is neutral during the catalytic cycle (pK_a < 5.5).[6]

NMR spectroscopy has proven to be an important method for directly determining the pK_a values of ionizable groups in protein side chains[12] and in the bases of RNA.[13] ^{13}C NMR experiments on UMP have shown that the three-bond H6−C2 coupling is significant (8.3 Hz)[14] and that a large upfield change in the ^{13}C chemical shift of C2 occurs upon protonation of N3 ($\Delta\delta$ = 7.9 ppm).[13] These observations suggested that a 2D ^{1}H−^{13}C long-range heteronuclear single quantum coherence (LR-HSQC) experiment could be used to determine the protonation and tautomeric states of the uracil base in the ternary UDG product complex.

Figure 2 shows the LR-HSQC spectra of [^{13}C]-2-uracil in the ternary UDG product complex[15] at pH values of 5.7, 6.3, and 7.5. The spectrum at pH 7.5 (Figure 2A) shows an H6−C2 correlation with a ^{13}C chemical shift of 164.7 ppm, which nearly matches the ^{13}C shift for free [^{13}C]-2-uracil at pH 10.2 when N1 and N3 are largely deprotonated (δ (C2) = 163.3 ppm, not shown). These comparable ^{13}C shifts, which are far from that of free uracil in the keto form at pH 7.5 (δ = 156.1 ppm, not shown), indicate that uracil is in the N1−O2 imidate form in the product complex at pH 7.5.[16] Using the Henderson−Hasselbalch equation and the observation that the keto form is present at ≤5% in this spectrum, an upper limit pK_a of 6.2 may be estimated for uracil N1. Accordingly, as the pH is lowered to 6.3, a second peak appears with nearly equal intensity and with a ^{13}C chemical shift

* Address correspondence to this author (J.T.S.). Telephone: 301-738-6264. FAX: 301-738-6255. E-mail: stivers@carb.nist.gov.

(1) David, S. S.; Williams, S. D. *Chem. Rev.* **1998**, *98*, 1221−61.
(2) (a) Zoltewicz, J. A.; Clark, F. D.; Sharpless, T. W.; Grahe, G. *J. Am. Chem. Soc.* **1970**, *92*, 1741−50. (b) Garrett, E. R.; Mehta, P. J. *J Am Chem Soc* **1972**, *94*, 8542−47.
(3) Shapiro, R.; Danzig, M. *Biochemistry* **1972**, *11*, 23−29.
(4) (a) Parikh, S. S.; Mol, C. D.; Slupphaug, G.; Bharati, S.; Krokan, H. E.; Tainer, J. A. *EMBO. J.* **1998**, *17*, 5214−26. (b) Xiao, G.; Tordova, M.; Jagadeesh, J.; Drohat, A. C.; Stivers, J. T.; Gilliland, G. L. *Proteins* **1999**, *35*, 13−24.
(5) Stivers, J. T.; Pankiewicz, K. W.; Watanabe, K. A. *Biochemistry* **1999,** *38*, 952−63.
(6) (a) Drohat, A. C.; Jagadeesh, J.; Ferguson, E.; Stivers, J. T. *Biochemistry* **1999**, *38*, 11866−11875. (b) Drohat, A. C.; Xiao, G.; Tordova, M.; Jagadeesh, J.; Pankiewicz, K. W.; Watanabe, K. A.; Gilliland, G. L.; Stivers, J. T. *Biochemistry* **1999**, *38*, 11876−11886.
(7) Drohat, A. C.; Stivers, J. T., unpublished results.
(8) Gerlt, J. A.; Gassman, P. G. *J. Am. Chem. Soc.* **1993**, *115*, 11552−11568.
(9) Shan, S. O.; Herschlag, D. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 14474−9.
(10) Luo, N.; Mehler, E.; Osman, R. *Biochemistry* **1999**, *38*, 9209−9220.
(11) Shroyer, M. J.; Bennett, S. E.; Putnam, C. D.; Tainer, J. A.; Mosbaugh, D. W. *Biochemistry* **1999**, *38*, 4834−45.
(12) Fersht, A. R. *Enzyme Structure and Mechanism*; W. H. Freeman & Co.: San Francisco, 1985.
(13) Legault, P.; Pardi, A. *J. Am. Chem. Soc.* **1997**, *119*, 6621−6628.
(14) Wijmenga, S. S.; van Buuren, B. N. M. *Prog. Nucl. Magn. Reson. Spectrosc.* **1998**, *32*, 287−387.
(15) The ds DNA used in these studies contained a tetrahydofuran abasic site analogue (Glen Research, Sterling, VA). Although this analogue differs slightly from the natural abasic product in that a 1′-hydrogen replaces the 1′-hydroxyl group, both abasic sites show the same highly deshielded resonance (δ ^{1}H = 15.6 ppm) arising from the hydrogen bond between His187 and uracil O2.[7] The DNA was synthesized, purified, and hybridized as previously described,[5] and the complementary oligonucleotides were 5′-GCGCA**X**-AGTCG-3′, and 5′-CGACTATGCGC-3′, where **X** denotes the abasic site.
(16) The possibility that we are measuring the ionization of N3 rather than N1 is negated by the fact that only a single ^{1}H−^{15}N correlation is observed in a conventional one-bond ^{1}H−^{15}N HSQC experiment conducted on the ternary UDG product complex with [$^{15}N_2$]-1,3-uracil (not shown). The ^{15}N chemical shift of this single peak (162.6 ppm) matches that of N3 (159.6 ppm) but not N1 (134.5 ppm) for free uracil in the keto form at pH 7.5 (not shown). Thus at pH 7.5 when C2 is in the imidate form, N3 of uracil has an attached proton and cannot participate in the formation of an N3−O2 imidate. The observation of the N3−H3 correlation is expected because this NH is hydrogen bonded to Asn123-O$^{\delta 1}$ as shown in Figure 1.[4]

10.1021/ja993254x CCC: $19.00
Published on Web 02/15/2000

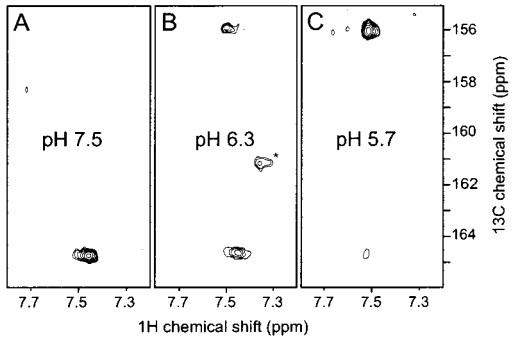

Figure 2. The $^1H-^{13}C$ LR-HSQC spectra showing the three-bond H6−C2 correlations of [^{13}C]-2-labeled uracil in the ternary UDG product complex at 25 °C at (A) pH 7.5, (B) pH 6.3, and (C) pH 5.7. A 2-fold excess of UDG and abasic DNA was used such that the uracil was completely bound[6] (1.0 mM UDG,[6] 1.1 mM ds abasic DNA,[15] 0.5 mM [^{13}C]-2-labeled uracil (Isotec, Inc., Miamisburg, OH), 10 mM NaH_2PO_4, 0.2 M NaCl, and 7% D_2O). The spectra were recorded on Bruker DRX500 and DRX600 NMR spectrometers using triple-axis gradient $^1H-^{13}C-^{15}N$ probes. ^1H chemical shifts are referenced to external DSS, and ^{13}C and ^{15}N chemical shifts are indirectly referenced to DSS and liquid NH_3, respectively.[23] The LR-HSQC spectra were collected using a conventional HSQC[24] pulse sequence with the de/rephasing delay set to 10.8 ms to eliminate signals from $^1J_{CH}$ couplings involving natural abundance ^{13}C at C5 and C6 of uracil. The spectra were recorded with ≥50 and 1024 complex points in t_1 and t_2, and acquisition times of ≥8.7 ms and ≥121 ms in t_1 and t_2, respectively. The ^1H and ^{13}C carrier positions were set on water and 155.35 ppm, respectively. The marked (*) correlation in (B) is not seen in the spectra of free uracil and probably arises from natural abundance ^{13}C in a highly mobile histidine ring (δ ^{13}C = 131.2 ppm). This peak is present in all of the spectra but appears folded at 161.2 ppm in (B) because the ^{13}C sweep width was 30 ppm for (B), rather than the 60 ppm used for (A) and (C).

of 155.9 ppm (Figure 2B). The nearly equal intensities and similar line widths (28 ± 3 Hz) of the two peaks observed at pH 6.3 indicates that (i) the N1 keto and imidate forms of uracil are in slow exchange,[17] (ii) that both forms are enzyme bound, and (iii) that the titration is about 50% complete. Finally, at pH 5.7 the intensity of the upfield peak corresponding to the keto form predominates and that of the imidate peak diminishes to essentially the background noise level (Figure 2C). A fit of the relative intensity of the imidate peak against pH yielded a $pK_a = 6.4 \pm 0.1$ for uracil N1 (Figure 3).

The surprising observation that uracil is in the N1−O2 imidate form in the UDG product complex at neutral pH requires that its pK_a is lowered by 3.4 units as compared to free uracil ($pK_a^{N1} = 9.8$).[18] This low pK_a indicates that the UDG active site stabilizes the negative charge on uracil O2 by 20 kJ/mol in the product complex. This stabilization energy is identical to the damaging effect in the transition-state that was found upon removal of His187 by directed mutagenesis, and is consistent with our proposal that the hydrogen bond between His187 and uracil O2 is worth about 20 kJ/mol in the transition-state.[6] The observation of similar stabilizing effects in the product- and transition-states

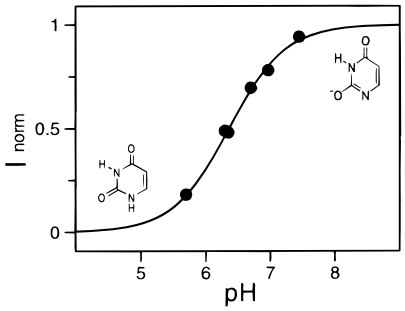

Figure 3. Determination of the N1 pK_a for uracil in the ternary UDG product complex. The $pK_a = 6.4 \pm 0.1$ was determined by a nonlinear fit of the normalized intensity (I_{norm}) of the imidate peak (δ ^{13}C = 164.7 ppm) to an ionization curve for a single proton using Grafit 4:[25] $I_{norm} = 10^{(pH-pKa)}/10^{(pH-pKa)} + 1$, where $I_{norm} = I_{\delta 164.7}/(I_{\delta 164.7} + I_{\delta 155.9})$. Normalized intensities were used to remove uncertainty due to changes in sample concentration during the pH titration, and because the imidate and keto forms of uracil are in slow exchange in the ternary product complex which excludes the possibility of following $\Delta\delta$ ^{13}C as a function of pH.

suggests a late transition state for glycosidic bond cleavage in which the N1−C1′ bond is largely broken and the negative charge has migrated to uracil O2.[19]

The pK_a of 6.4 for uracil N1 matches the apparent pK_a we have recently measured by ^1H NMR for the disappearance of a deshielded proton that participates in a hydrogen bond between His187 and uracil O2,[7] which indicates that a negative charge on uracil O2 is *required* to form this hydrogen bond. The low pK_a for the imidazolium−imidazole ionization of His187 (<5.5)[6] suggests that the pK_a for the imidazole−imidazolate ionization may also be lower than free imidazole,[20] which would help minimize the difference in pK_a between His187-N$^{\epsilon 2}$ and uracil O2 imidol and enhance the energetics of this interaction.[9,21] Taken together, these NMR results are inconsistent with two recent proposals that the developing negative charge in the transition state resonates significantly to uracil O4[11] and that His187 is cationic in the transition state.[10] We therefore propose a general mechanism for leaving-group activation by pyrimidine-specific glycosylases which involves a preorganized active site that is highly evolved to "solvate" the developing negative charge on the oxygen atoms in the transition state as nucleophilic attack at C1′ proceeds. Thus, unfavorable solvent reorganization in the transition-state may be a factor in the slow rates of hydrolysis of pyrimidine nucleosides in aqueous environments. Whether this hydrogen bond meets the criteria proposed for a low barrier hydrogen bond[22] is currently being investigated.

Acknowledgment. This work was supported by NIH grant GM56834 (J.T.S.). A.C.D. is a National Research Council Postdoctoral Research Associate.

JA993254X

(17) From the difference in ^{13}C chemical shift of the neutral and anionic species under slow exchange conditions, an upper limit exchange rate of 2400 s^{-1} can be calculated for the N1 proton from the relationship $k_{ex} = \pi\delta\nu/\sqrt{2}$ (Hore, P. J. *Nuclear Magnetic Resonance*; Oxford University Press Inc.: New York, 1995; p 47). This is about 1000-fold greater than the k_{cat} value for duplex DNA, which is limited by the off-rate of the products,[5] which suggests that k_{off} may limit k_{ex}. It is unlikely that the enzyme *selectively* binds the N1-deprotonated tautomer of free uracil from solution because the NMR spectrum in Figure 2B shows that both anionic and neutral forms are bound at pH 6.3.

(18) Shapiro, R.; Kang, S. *Biochemistry* **1969**, *8*, 1806−10. The observed pK_a of 9.45 for free uracil is a composite of the overlapping ionizations of the N1 and N3 positions. The equilibrium (K_T) between the prototropic tautomers (N1/N3−H ↔ N1−H/N3) was previously determined, and the N1/N3−H tautomer is present to the extent of 49%. The $pK_a = 9.76$ for N1 of free uracil was calculated from the relationships $K_A = K_1 + K_3$, and $K_T = K_1/K_3$, where K_1 and K_3 are the equilibrium constants for ionization of N1 and N3, respectively.

(19) Werner, R. M.; Stivers, J. T. Unpublished results. Competitive kinetic isotope effect studies on the UDG reaction indicate a large 1′-^2H KIE of 1.158 ± 0.004, and a small primary 1′-^{13}C KIE of 1.005 ± 0.013, consistent with a late oxycarbenium ion transition state in which the N1−C1′ bond is largely broken.

(20) Bruice, T. C.; Schmir, G. L. *J. Am. Chem. Soc.* **1958**, *80*, 148−156.

(21) NMR measurements indicate that the imidazole-imidazolate pK_a of His187 in the free enzyme and the product complex is higher than 10.5.[6,7] A possible mechanism by which UDG could bring the pK_a values for the uracil O2 imidol and His187-N$^{\epsilon 2}$ closer together is to increase the N1−O2 keto → imidol tautomerization constant of bound neutral uracil (pK_T), according to the thermodynamic relationship $pK_aO2 = pK_aN1 - pK_T$ (see Chiang, Y.; Kresge, A. J. *Science* **1991,** *253*, 395−400). This possibility is being tested.

(22) Mildvan, A. S.; Harris, T. K.; Abeygunawardana, C. *Methods Enzymol.* **1999**, *308*, 219−245.

(23) Wishart, D. S.; Bigam, C. G.; Yao, J.; Abildgaard, F.; Dyson, H. J.; Oldfield, E.; Markley, J. L.; Sykes, B. D. *J. Biomol. NMR* **1995**, *6*, 135−40.

(24) Mori, S.; Abeygunawardana, C.; Johnson, M. O.; van Zijl, P. C. *J. Magn. Reson. B* **1995**, *108*, 94−8.

(25) Leatherbarrow, R. J. *GraFit 4.0*; Erithacus Software Ltd.: Staines, U.K., 1998.