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Acidity and Proton Affinity of Hypoxanthine in the Gas Phase versus in Solution: Intrinsic Reactivity and Biological Implications

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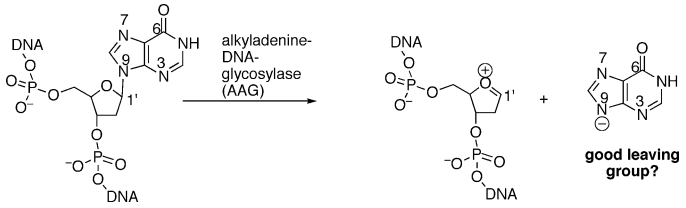
Received May 10, 2007



Hypoxanthine is a mutagenic purine base that most commonly arises from the oxidative deamination of adenine. Damaged bases such as hypoxanthine are associated with carcinogenesis and cell death. This inevitable damage is counteracted by glycosylase enzymes, which cleave damaged bases from DNA. Alkyladenine DNA glycosylase (AAG) is the enzyme responsible for excising hypoxanthine from DNA in humans. In an effort to understand the intrinsic properties of hypoxanthine, we examined the gas-phase acidity and proton affinity using quantum mechanical calculations and gas-phase mass spectrometric experimental methods. In this work, we establish that the most acidic site of hypoxanthine has a gas-phase acidity of 332 ± 2 kcal mol^{-1}, which is more acidic than hydrochloric acid. We also bracket a less acidic site of hypoxanthine at 368 ± 3 kcal mol^{-1}. We measure the proton affinity of the most basic site of hypoxanthine to be 222 ± 3 kcal mol^{-1}. DFT calculations of these values are consistent with the experimental data. We also use calculations to compare the acidic and basic properties of hypoxanthine with those of the normal bases adenine and guanine. We find that the N9-H of hypoxanthine is more acidic than that of adenine and guanine, pointing to a way that AAG could discriminate damaged bases from normal bases. We hypothesize that AAG may cleave certain damaged nucleobases as anions and that the active site may take advantage of a nonpolar environment to favor deprotonated hypoxanthine as a leaving group versus deprotonated adenine or guanine. We also show that an alternate mechanism involving preprotonation of hypoxanthine is energetically less attractive, because the proton affinity of hypoxanthine is less than that of adenine and guanine. Last, we compare the acidity in the gas phase versus that in solution and find that a nonpolar environment enhances the differences in acidity among hypoxanthine, adenine, and guanine.

Introduction

The acidities and the basicities of nucleobases and nucleobase derivatives are germane to several biological issues. First, the recognition of DNA and RNA bases is modulated by hydrogen bonding; in turn, hydrogen bonding is correlated to the intrinsic acidity and basicity of acceptor and donor groups on the nucleobases. Second, elucidating the intrinsic reactivity of nucleobases can improve understanding of key biosynthetic mechanisms for which those nucleobases are substrates.[1−14]

The gas phase is a particularly valuable environment in which to examine the properties and reactivity of biological molecules. Biological media, from intracellular environs to the interior of

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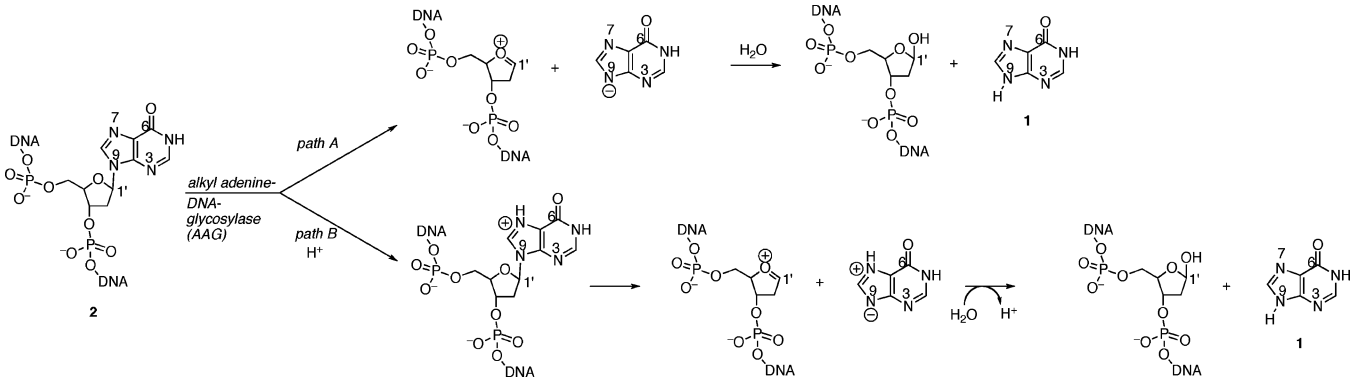
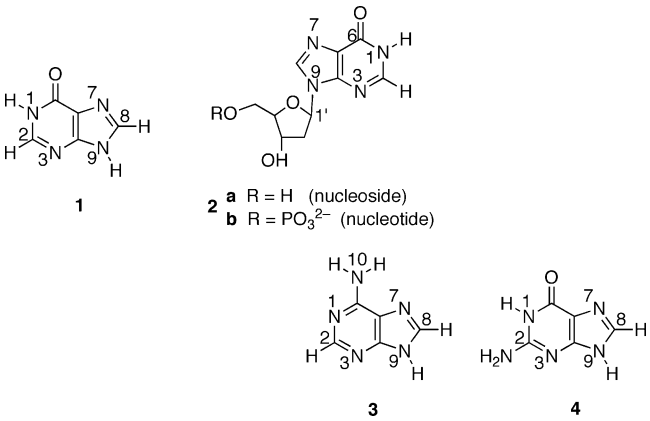
10.1021/jo070996x CCC: $37.00
Published on Web 07/27/2007

SCHEME 1



proteins, are seldom aqueous in nature.[7,15,16] The gas phase is the "ultimate" nonpolar environment, where intrinsic reactivity can be explored and extrapolated to other media.[8−13,17−19]

Recently, our studies of nucleobases have focused on the mutated purine base hypoxanthine (**1**).[20] While hypoxanthine is in fact a naturally occurring base—in its nucleotide form (called "inosine" (**2**)), it is a key intermediate in the de novo biosynthesis of purine nucleotides—it is also a damaged base that is formed in DNA when adenine (**3**) undergoes oxidative deamination.[20−25] The mutagenicity of hypoxanthine is believed to arise from the fact that unlike adenine, which hydrogen bonds to thymine in the double helix, hypoxanthine prefers to hydrogen bond with cytosine.[23−27] This mispairing when propagated is deleterious to the genome and unless repaired, could lead to cancer or cell death.[28]



As with many damaged bases, hypoxanthine is cleaved from DNA via a genome-protecting reaction catalyzed by an enzyme called a glycosylase.[25,27,29−34] In human cells, alkyladenine DNA glycosylase (AAG) effects this cleavage.[29−32,34−41] AAG is particularly interesting because it cleaves a broad range of bases yet does not cleave normal bases; the conundrum is how the enzyme achieves this "broad specificity".[28,29,31,32] Most glycosylases, like uracil DNA glycosylase (UDGase), are specific to one nucleobase (in the case of UDGase, the enzyme will cleave only uracil).[28,29,31,32] However, AAG is specific yet broad, cleaving a variety of alkylated purines, including 3-methyladenine, 7-methylguanine, and 1, N^6-ethenoadenine in addition to hypoxanthine, yet leaving normal bases (adenine (**3**) and guanine (**4**)) untouched.[28,31,32,42]

In terms of mechanism, one can imagine cleavage with "deprotonated hypoxanthine" as the leaving group (Scheme 1A), or protonation of the hypoxanthine first to facilitate cleavage (Scheme 1B).[28,32,43] Little is known about the catalytic mechanism of AAG, but pH rate profiles imply that AAG-catalyzed excision of hypoxanthine requires both a general base (Glu 125)

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and a general acid. Mutation experiments have thus far failed to identify which residue is the general acid catalyst.[28,30,32]

We have found that gas-phase reactivity can be extremely intriguing and lend insight into glycosylase mechanisms.[8,9,12,13] In previous studies, we showed that the *gas phase* acidities of uracil (a mutation when present in DNA) and 3-methyladenine are very high, leading to a prediction that relatively speaking, the deprotonated anions of these bases would be good leaving groups and the nonpolar enzyme active sites could take advantage of this property for facile cleavage.[8,12] We also found that relative acidities change in the gas phase versus solution; for example, while the two "NH" groups in uracil have the same pK_a in solution, they have very different acidities in the gas phase, with the N1-H being 15 kcal mol^{-1} more acidic than the N3-H.[8] This raises the interesting possibility that a nonpolar environment could be used to enhance discrimination between sites or substrates.

This leads to the question of the intrinsic, gas-phase reactivity of hypoxanthine. The gas-phase acidity of hypoxanthine at N9 should correlate to its leaving group ability in a nonpolar active site; if the hypoxanthine N9 proton is unusually acidic, this could be a path by which AAG might favor cleavage of damaged bases over normal bases. We were interested to probe whether hypoxanthine would have enhanced acidity relative to normal nucleobases (adenine and guanine) and whether the relative acidities change in solution versus the gas phase.[8,9,12−14] Second, the gas-phase proton affinity of hypoxanthine is also of interest, since proton transfer may precede cleavage; again, we sought to uncover the differences in the gas phase versus solution. In this paper, we describe a thorough gas-phase computational and experimental examination of hypoxanthine, providing the first measurements of the acidic and basic properties of this damaged base, followed by a discussion of both the intrinsic properties of this nucleobase and also how those properties relate to the biological mechanism of the enzyme human alkyladenine DNA glycosylase (AAG).

Results

Computational Results: Tautomers. The structure of nucleobases is such that several tautomers are often possible, and hypoxanthine is no exception. We calculated the relative stability of the possible tautomers of hypoxanthine. The canonical structure **1**, which we refer to as the "H19" tautomer (since the protons reside on the N1 and the N9) is calculated to be *less* stable than the "H17" tautomer (**5**, where the protons reside on the N1 and the N7) by 0.8 kcal mol^{-1} (Figure 1). The next most stable tautomer is **6**; at 5.4 kcal mol^{-1} less stable than the H17 tautomer, it is not likely to be present in any appreciable amount in the gas phase. The remaining possible tautomers are even less stable (Figure S1, Supporting Information).

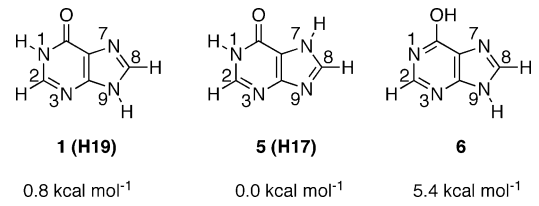

FIGURE 1. Relative energies (ΔH) of the three most stable tautomers of hypoxanthine, calculated at B3LYP/6-31+G*, at 298 K.

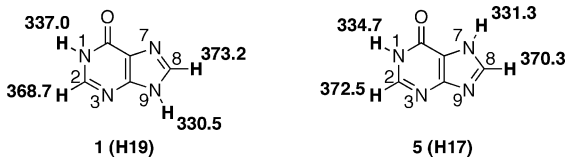

FIGURE 2. Calculated acidities (ΔH_{acid}) of the two most stable tautomers of hypoxanthine at B3LYP/6-31+G*, at 298 K. Acidic protons are in bold.

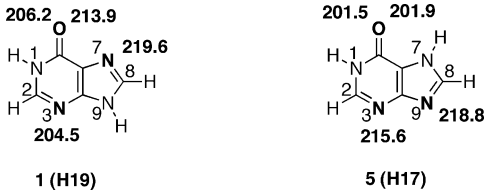

FIGURE 3. Calculated proton affinities (ΔH) of the two most stable tautomers of hypoxanthine at B3LYP/6-31+G*, at 298 K. Basic sites are in bold.

Computational Results: Acidity. We focused our acidity studies on the two most stable tautomers, H19 (**1**) and H17 (**5**). The H19 tautomer is the "canonical" structure and the most relevant biologically. The H17 structure is of importance intrinsically, since its high stability is such that it might be present in our gas-phase studies.

The gas-phase acidities for all the protons of hypoxanthine calculated at B3LYP/6-31+G* are summarized in Figure 2. The most acidic site of the H19 tautomer **1** is the N9-H, at 330.5 kcal mol^{-1}. The N1-H is slightly less acidic, at 337.0 kcal mol^{-1}. The C-H protons are the least acidic: the C2-H calculates to 368.7 kcal mol^{-1} and the C8-H to 373.2 kcal mol^{-1}.

The H17 tautomer **5** is also quite acidic. The N7-H calculates to 331.3 kcal mol^{-1} while the N1-H is slightly less acidic, at 334.7 kcal mol^{-1}. The least acidic sites are the C-H protons: the C2-H calculates to 372.5 kcal mol^{-1} and the C8-H to 370.3 kcal mol^{-1}.

Computational Results: Proton Affinity. The proton affinities of the tautomers H19 and H17 were also explored computationally (Figure 3). The H19 tautomer has several heteroatoms that could accept a proton; we find the most basic site to be N7, at 219.6 kcal mol^{-1}. The O6 site is the next most basic, with a PA of 213.9 kcal mol^{-1} (for the protonated structure where the proton is "pointing toward" the imidazolic ring). Protonation of O6 to form the structure where the proton is on the same side as the N1-H is exothermic by 206.2 kcal mol^{-1}, and the least basic heteroatom on the H19 tautomer is N3, at 204.5 kcal mol^{-1}. The H17 tautomer is of similar overall basicity (as compared to the H19); the most basic site is N9, at 218.8 kcal mol^{-1}. The remaining proton affinities are 215.6 (N3), 201.9 (O6, imidazole (N7) side), and 201.5 (O6, N1 side) kcal mol^{-1}.

(42) When alkylated, adenosine and guanosine form positively charged substrates (3-methyladenosine and 7-methylguanosine); these may bind to the AAG active site as charged substrates. This paper focuses on AAG substrates such as hypoxanthine and ethenoadenine, which are believed to bind as neutrals. See refs 28−32.

(43) Previous work indicates that glycosidic bond hydrolyses proceed via a highly dissociative S_N2 or a stepwise S_N1 mechanism; to simplify Scheme 1 we show the mechanism as S_N1. Furthermore, the mechanism for enzyme cleavage also involves a "base-flipping" step, whereby the nucleobase is "flipped" into the enzyme active site; this step may also differ for different substrates. It is not known whether base flipping is a relatively fast or slow step in AAG, and herein we focus on the steps involving actual nucleobase excision. See refs 28−30.

TABLE 1. Summary of Results for Acidity Bracketing of the More Acidic Site of Hypoxanthine

reference compd	ΔH_{acid}[a]	proton transfer[b] ref acid	proton transfer[b] conjugate base
HCOOH	346.2 ± 1.2	−	+
$CH_3COCH_2COCH_3$	343.8 ± 2.1	−	+
m-CF_3PhOH	339.3 ± 2.1	−	+
$CH_3CHBrCOOH$	336.8 ± 2.1	−	+
$CNCH_2CN$	335.8 ± 2.1	−	+
HCl	333.4 ± 0.1	−	+
$(CF_3)_3COH$	331.6 ± 2.2	+	−
CHF_2COOH	331.0 ± 2.2	+	−
$(CF_3)_2C_6H_3OH$	329.8 ± 2.1	+	−
$CF_3COCH_2COCH_3$	328.3 ± 2.9	+	−

[a] Acidities are in kcal mol^{-1} and come from ref 44. [b] A "+" indicates the occurrence and a "−" denotes the absence of proton transfer.

TABLE 2. Summary of Results for Acidity Bracketing of the Less Acidic Site of Hypoxanthine

reference compd	ΔH_{acid}[a]	proton transfer[b] ref acid
$CH_3CH_2CH_2OH$	375.7 ± 1.3	−
CH_3CN	372.9 ± 2.1	−
$PhCH_2OH$	370.0 ± 2.1	−
CH_3COCH_3	368.8 ± 2.0	−
$CH_3COCH_2CH_3$	367.2 ± 2.8	+
$C_6H_5NH_2$	366.4 ± 2.1	+
m-F-$PhNH_2$	362.6 ± 2.2	+
C_4NH_5	359.5 ± 0.3	+
CH_3COOH	348.1 ± 2.2	+
$CH_3CH_2CH_2CH_2COOH$	346.2 ± 2.1	+
HCOOH	346.2 ± 1.2	+

[a] Acidities are in kcal mol^{-1} and come from ref 44. [b] A "+" indicates the occurrence and a "−" denotes the absence of proton transfer.

Experimental Results: Acidity. Our first step was to bracket the most acidic site of hypoxanthine (Table 1). We find that the conjugate base of hypoxanthine deprotonates $(CF_3)_3COH$ (ΔH_{acid} = 331.6 ± 2.2 kcal mol^{-1}), but not HCl ((ΔH_{acid} = 333.4 ± 0.1 kcal mol^{-1}); also, Cl^- deprotonates hypoxanthine but $(CF_3)_3CO^-$ does not. We therefore bracket the most acidic site of hypoxanthine to be 332 ± 2 kcal mol^{-1}. We next used our "less acidic" method (Table 2). We find that the conjugate base of hypoxanthine does not deprotonate acetone (CH_3COCH_3, ΔH_{acid} = 368.8 ± 2.0 kcal mol^{-1}) but it does deprotonate methyl ethyl ketone ($CH_3COCH_2CH_3$, ΔH_{acid} = 367.2 ± 2.8 kcal mol^{-1}). Therefore, we bracket the gas-phase acidity of the less acidic site in hypoxanthine to be 368 ± 3 kcal mol^{-1}. At this point, we do not know which tautomer(s) or which sites we are bracketing; we simply were interested as a first step to bracket the experimental acidity values.

Experimental Results: Proton Affinity. We next measured the most basic site of hypoxanthine. We find that 3-bromopyridine (PA = 217.5 ± 2.0 kcal mol^{-1}) cannot deprotonate protonated hypoxanthine, but that hypoxanthine can protonate 3-bromopyridine. 1-Butylamine (PA = 220.2 ± 2.0 kcal mol^{-1}) deprotonates protonated hypoxanthine, while the reverse reaction does not occur. In between these two reference bases, we obtain some conflicting results. For example, while the reference base benzylamine (PA = 218.3 ± 2.0 kcal mol^{-1}) deprotonates protonated hypoxanthine, *N*-methylaniline (PA = 219.1 ± 2.0 kcal mol^{-1}) *cannot* deprotonate protonated hypoxanthine. The ambiguity of our bracketing results may be due to the error (about ±2 kcal mol^{-1}) in any given PA value; from our data, we only can say that the PA of hypoxanthine is between 217.5-(±2) and 220.2(±2) kcal mol^{-1}.[44]

Given the large range of our bracketing result, we decided to measure the PA using a second method, the Cooks extended kinetic method (see the Experimental Section for details). Four reference bases were used: 1-octanamine (PA = 222.0 kcal mol^{-1}), isobutylamine (PA = 221.0 kcal mol^{-1}), 1-butylamine (220.2 kcal mol^{-1}), and 1-propylamine (219.4 kcal mol^{-1}), yielding a proton affinity of 222 ± 3 kcal mol^{-1} for hypoxanthine, which is consistent (though on the high end) with the window that we find via bracketing.[45−48]

The measurement of the less basic site of hypoxanthine had experimental issues that we could not overcome. In essence, under the "less basic" FTMS conditions, we always see proton transfer from protonated hypoxanthine to the reference base, even if the reference base is very weak (such as acetone, PA = 194.1 kcal mol^{-1}). We have experienced this problem in the past, and it appears to be due to the presence of protonated water, which is very acidic.[10] We tried various methods to rid the system of H_3O^+, but to no avail, and therefore could not measure the less basic site.

Discussion

In aqueous solution and in the solid state, the canonical H19 (**1**) tautomer of hypoxanthine predominates.[49−51] Furthermore, this particular tautomer is the biologically relevant one; when hypoxanthine is in nucleotide form ("inosine" (**2**)), the deoxyribose moiety is attached to the N9; therefore, the H19 tautomer is a model for the nucleobase portion of inosine.

Unlike in solution and in the solid state, more than one tautomer is accessible in the gas phase. Our calculations at B3LYP/6-31+G* indicate that the H19 structure (**1**) is actually less stable than the H17 structure (**5**) by 0.8 kcal mol^{-1} (Figure 1), which is consistent with other calculations on hypoxanthine tautomers.[50−56] In this Discussion section, we will first discuss

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(45) The $\Delta\Delta S$ value obtained for hypoxanthine from our Cooks extended kinetic method experiments is 5 cal K^{-1} mol^{-1}. It has been noted that the $\Delta\Delta S$ value is related to the accuracy of the PA value obtained by the Cooks extended method. Ideally, the actual $\Delta\Delta S$ value should be less than or equal to about 5 cal K^{-1} mol^{-1}; otherwise, the extended kinetic method may underestimate the PA. Another caveat is that the $\Delta\Delta S$ value obtained from the extended method is often itself underestimated. There is therefore a possibility that the PA value of 222 kcal mol^{-1} obtained via our Cooks extended method experiment is too low; however, given that the bracketing result gives a PA of 217.5−220.2 kcal mol^{-1}, we are inclined to believe that the extended kinetic method value is not highly underestimated.

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the intrinsic gas-phase properties of hypoxanthine, in which we consider both the H19 (**1**) and H17 (**5**) tautomers. We will then move on to discuss the biological relevance of our experimental and computational results, which will focus on the H19 canonical tautomer **1**.

Can We Differentiate between Tautomers? The acidity and proton affinity calculations indicate that measurements of these properties are not likely to reveal which tautomer(s) is (are) present. That is, sometimes two tautomers have very differing properties; one tautomer might be much more acidic than the other, and the measurement of the acidity can thus reveal which tautomer is present. In the case of hypoxanthine, however, the most acidic site of the H19 tautomer is calculated to be 330.5 kcal mol^{-1}, while the most acidic site of the H17 tautomer calculates to 331.3 kcal mol^{-1}. These acidities are so close that any measured value could be attributable to either tautomer. For proton affinity (PA), the most basic site of the H19 tautomer has a calculated PA of 219.6 kcal mol^{-1}, while the most basic site of the H17 tautomer has a calculated PA of 218.8 kcal mol^{-1}. Again, these values are sufficiently close that the actual measurement will not divulge which tautomer has been probed. It may be that the more stable H17 tautomer is prevalent and that our measurements pertain to that structure or a mix of H17 and H19.

We measured two acidic sites on hypoxanthine, one at 332 $\pm$ 2 kcal mol^{-1} and the other at 368 $\pm$ 3 kcal mol^{-1} (Tables 1 and 2). The more acidic site is consistent with the calculated values for the most acidic site of both the H19 and the H17 tautomers (330.5 (N9-H of H19 tautomer) and 331.3 kcal mol^{-1} (N7-H of H17 tautomer), Figure 2). Either or both tautomers may be present; calculations would indicate that in the gas phase, we have a mixture of both tautomers.[50,53]

The "less acidic" bracketing experiment yields a value of 368 $\pm$ 3 kcal mol^{-1}. By calculations, however, the next most acidic site on the H19 (**1**) tautomer is the N1-H, at 337.0 kcal mol^{-1} (Figure 2). On the H17 (**5**) tautomer, the second most acidic site is also the N1-H, at 334.7 kcal mol^{-1} (Figure 2). Our "less acidic" bracketing method is such that if we have a mixture of $[M-H]^-$ ions deprotonated at N1 as well as at C2 and C8, we will only see the acidity value corresponding to the most basic anions (in this case, the carbanions).[8,9,12,13,57] Therefore, we may have ions resulting from the N1-H of the H17 tautomer and/or the N1-H of the H19 tautomer, but we can only bracket the most basic ion due to the nature of the experiment.[8−10,12,13] Given how close in energy both tautomers are calculated to be, we believe that we are likely to have a tautomer mixture.[50−56] Therefore, the less acidic site of 368 $\pm$ 3 kcal mol^{-1} could correspond to any of the C-H sites in the H19 (**1**) and/or the H17 (**5**) tautomers (Figure 2).

For proton affinities, we find the more basic site to have a PA of 222 $\pm$ 3 kcal mol^{-1}. This value is consistent with the most basic calculated sites (Figure 3) of the H19 (**1**) tautomer (N7, 219.6 kcal mol^{-1}), and the H17 (**5**) tautomer (N9, 218.8 kcal mol^{-1}). The PA results thus also correlate with the computational results.

These acidities and proton affinities of hypoxanthine are the first such measurements and serve to both benchmark the calculations as well as provide insights into the intrinsic reactivity of this damaged base.

Biological Implications. As noted earlier, one of our interests in hypoxanthine is that it is a mutagenic base. When incorporated into DNA, hypoxanthine is called "inosine" (**2**). As shown in Scheme 1, hypoxanthine is excised from DNA via scission of the N9−C1′ bond; the relevant tautomer is therefore the H19 (**1**), and the discussions herein will focus solely on this canonical tautomer. Our genome is protected by an enzyme called alkyladenine DNA glycosylase (AAG), which cleaves hypoxanthine from DNA. AAG is a particularly intriguing enzyme because it cleaves a wide range of damaged bases, thus achieving a "broad specificity".[28,29,31,32] One of the puzzles is how an enzyme could cleave *so many* different bases—in this case, a variety of alkylated purines, including 3-methyladenine, 7-methylguanine, and 1, N^6-ethenoadenine in addition to hypoxanthine—yet leave normal bases untouched. The exact mechanism by which AAG excises hypoxanthine is unknown.[29−32,34−41] Two possibilities are that the hypoxanthine is excised in an anionic state or that it is protonated prior to departing as a neutral (Scheme 1). Our results lend insight into both possibilities.

Deprotonated Hypoxanthine as a Leaving Group. Should hypoxanthine undergo cleavage without prior protonation, the N9-deprotonated hypoxanthine would be the leaving group (Scheme 1A). The more acidic the N9-H, the more easily deprotonated hypoxanthine should be cleaved. Our prior studies have shown that the *gas-phase* acidities of uracil (an undesirable base in DNA) and 3-methyladenine (a mutated base) are very high, leading to a prediction that relatively speaking, the deprotonated anions of these damaged bases would be good leaving groups in nonpolar enzyme active sites (since the gas phase is the ultimate nonpolar environment), which in turn translates to facile cleavage.[8,9,12,13,58] Furthermore, recently, Drohat and co-workers published a thorough study of a *pyrimidine* glycosylase that also has broad specificity, human thymine DNA glycosylase (TDG).[14] Their studies show a relationship between the solution phase acidity (pK_a) of a series of nucleobase substrates and the ease of excision of these substrates by the enzyme TDG (the more acidic the nucleobase, the more facile the excision), indicating the nucleobase is cleaved in its deprotonated form. In addition, the differences in acidity among the substrates appear to be much greater in a nonpolar gas-phase environment than in solution. That is, the nonpolarity of the active site of TDG appears to contribute to the enzyme's specificity by enhancing the differences in acidity of the various substrates and therefore favoring cleavage of those nucleobases that have high acidities (and are therefore good deprotonated leaving groups) in the gas phase.[14]

The gas-phase acidity of hypoxanthine at N9 should likewise indicate its leaving group ability in a nonpolar active site. The calculated acidity of the N9-H of hypoxanthine (**1**) at B3LYP/6-31+G* is 330.5 kcal mol^{-1}. The acidity of the N9-H of guanine (**4**) calculated at the same level is 334.3 kcal mol^{-1}.[59−62] That is, hypoxanthine is ~4 kcal mol^{-1} more acidic than guanine in the gas phase. This enhanced acidity of hypoxanthine would

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translate to its ease of excision; AAG, like TDG, may capitalize on a nonpolar enzyme active site to favor cleavage of damaged bases like hypoxanthine due to their better leaving group ability. The gas-phase acidity of hypoxanthine is also calculated to be 4 kcal mol^{-1} less (i.e., more acidic) than that of adenine (hypoxanthine (**1**), 330.5 kcal mol^{-1} versus adenine (**3**), 334.8 kcal mol^{-1}).[12,13,59−61,63] Thus, the enhanced acidity of the damaged base hypoxanthine relative to the normal bases adenine and guanine would render it more easily excised by AAG; this provides an explanation for why AAG might favor hypoxanthine for removal.

We were also interested in ascertaining whether the acidity of hypoxanthine is enhanced, relative to adenine and guanine, in the gas phase versus in solution.[14] The solution-phase acidities (pK_a) of the N9-H of adenine, guanine, and hypoxanthine are reported to be 9.8, 10.0, and 8.9, respectively.[51,64−67] These pK_a values translate to deprotonation at 298 K being more favorable for hypoxanthine by 1.2 kcal mol^{-1} over adenine, and 1.5 kcal mol^{-1} over guanine. This preference is enhanced in the gas phase, where the acidity of hypoxanthine is calculated to be 4.3 and 3.8 kcal mol^{-1} greater than that of adenine and guanine, respectively. A nonpolar environment would therefore serve to enhance the differences among the substrates adenine, guanine, and hypoxanthine, and favor cleavage of the damaged base hypoxanthine. We hypothesize that one of the ways AAG provides selectivity is by targeting hypoxanthine and other damaged bases and cleaving them as deprotonated, anionic nucleobases.

We also conducted dielectric medium calculations on the acidities of hypoxanthine, adenine, and guanine to determine how the gas-phase acidities change in a medium of dielectric 78.4 (aqueous solution). We find that the results are consistent with trends seen with the experimental pK_a values. The calculated acidity of hypoxanthine in an aqueous continuum is 295.7 kcal mol^{-1}; those of adenine and guanine are 297.8 and 298.4 kcal mol^{-1}. Therefore, hypoxanthine is still the most acidic, but less so than in the gas phase (more acidic than adenine and guanine respectively by only 2.1 and 2.7 kcal mol^{-1} in a water dielectric versus 4.3 and 3.8 kcal mol^{-1} in the gas phase). Therefore, the gas-phase and solvation calculations and the experimental pK_a data all indicate that hypoxanthine is more acidic than adenine and guanine, and that the differences in acidity are greatest in the gas phase.

Neutral Hypoxanthine as a Leaving Group. Should hypoxanthine leave as a neutral, the proton affinity is relevant: the more basic hypoxanthine is, the more easily it will be protonated (Scheme 1B). Assuming that once protonation occurs, cleavage is facile, then protonation would be a rate-determining step.[29,30] The proton affinity of the most basic site of the H19 tautomer of hypoxanthine (**1**, which is relevant to the biological compound inosine (**2**) since the ribose is attached to N9, Scheme 1B) is calculated to be 219.6 kcal mol^{-1}, at B3LYP/6-31+G*. We calculate the proton affinities of adenine (**3**) and guanine (**4**) to be, using the same method, respectively 223.7 and 227.4 kcal mol^{-1}.[60,61,68−70] Therefore, hypoxanthine appears to be *less basic* than adenine and guanine. A mechanism involving protonation prior to cleavage (Scheme 1B) would not energetically favor hypoxanthine cleavage. This trend is mitigated, but still exists, in solution. The solution-phase proton affinity of hypoxanthine is still the lowest (pK_a of protonated hypoxanthine is 2.0, whereas those of protonated adenine and protonated guanine are 4.2 and 3.3, respectively). Although as with acidity, the proton affinity differences among the substrates is less in solution than in the gas phase, a mechanism involving protonation still does not favor hypoxanthine and therefore does not serve as an explanation for how the damaged base hypoxanthine would be selected over normal bases.[71]

Of course, AAG might also differentiate hypoxanthine from normal bases through other means (binding energetics, size selectivity),[28,31,32] but our results show that energetically speaking, a mechanism involving initial proton transfer does not favor the damaged base. We therefore propose the possibility that like thymine DNA glycosylase (TDG), hypoxanthine is excised by AAG as an anion, and that hypoxanthine should be a better leaving group (N9-H is more acidic) than the normal bases adenine and guanine.[8,9,12−14] Furthermore, this trend is enhanced in the gas phase, and would therefore be enhanced in a nonpolar active site. We look forward to experimental testing of this hypothesis.

Conclusions

We have established the acidities and proton affinity of the damaged nucleobase hypoxanthine. The experimental studies allow us to confirm the accuracy of the ab initio calculations. The acidic and basic properties of hypoxanthine are compared to those of the normal nucleobases adenine and guanine, both in the gas phase and in solution, to provide insights into understanding how the enzyme alkyladenine DNA glycosylase (AAG) might discriminate damaged bases from normal bases. The results highlight the possibility that AAG cleaves damaged nucleobases as anions and that the active site may take advantage of a nonpolar environment to favor deprotonated hypoxanthine as a leaving group versus deprotonated adenine or guanine. Future studies of the gas-phase properties of other damaged nucleobases that serve as substrates for AAG are underway.

Experimental Section

All chemicals are commercially available and were used as received. Experiments were conducted on a Fourier Transform mass spectrometer (FTMS) with a dual cell setup that has been described

(62) Note that in ref 59, the calculated guanine acidity at B3LYP/6-31+G* is reported as 334.8 kcal mol^{-1}. We are not sure why their results differ slightly from ours, but the difference is very small and does not change the conclusions herein.

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(71) Intriguingly, the PA values of adenine and guanine reverse in solution versus the gas phase. Adenine is more basic than guanine in solution (respective pK_a values of 4.2 and 3.3), but less so in the gas phase (respective PA values of 223.7 and 227.4 kcal mol^{-1}). The same trend is seen in aqueous medium dielectric calculations (adenine is more basic than guanine: adenine PA, 283.8 kcal mol^{-1}; guanine PA, 282.4 kcal mol^{-1}). In all cases, hypoxanthine is the least basic (pK_a, 2.0; solvent dielectric medium acidity, 279.6 kcal mol^{-1}; gas phase calculated acidity, 219.6 kcal mol^{-1}). Future work will probe this adenine-guanine gas−solution phase trend reversal.

previously.[8,12] Briefly, the setup consists of two adjoining 2 in. cubic cells that are pumped to a baseline pressure of less than 1×10^{-9} Torr. The dual cell is positioned colinearly with the magnetic field produced by a 3.3 T superconducting magnet. A heated batch inlet system or a heated solids probe is used to introduce neutral samples into the FTMS. A trapping potential of 2 V (positive if positive ions are being trapped and negative if negative ions are being trapped) is applied to the cell walls perpendicular to the magnetic field at all times except when ions are being transferred from one cell to another. Transfer is accomplished by temporarily grounding (40−150 μs) the trapping plate separating the two cells. The ions can then be transferred into the next cell through a 2-mm hole in the center of the trapping plate. Transferred ions are cooled by a pulse of argon that raises the cell pressure to 10^{-5} Torr.[72,73]

Bracketing methods were utilized to measure the gas-phase acidities and basicities (proton affinities). Hydroxide ions are generated by pulsing water into the FTMS cell and sending an electron beam (typically 8 eV, 6 μA, beam time 0.5 s) through the center of the cell. Hydronium ions are also generated via pulsing water and an electron beam (20 eV, 6 μA, beam time 0.2 s). Hydroxide is used to deprotonate the molecules of interest, producing for example, the $[M-H]^-$ anion of hypoxanthine. The $[M-H]^-$ anions are transferred into the second cell and allowed to react with reference acids having known gas-phase acidities.[44] For acidity bracketing of the most acidic site in a molecule, we also allow the conjugate bases of different reference acids to react with the neutral of unknown acidity. Rapid proton transfer (i.e., near the collision rate) was taken as evidence that the reaction was exothermic and is indicated by a "+" in Tables 1 and 2.[74] The same procedure is used for proton affinity bracketing (wherein hydronium ions are used for protonation).

We have recently developed an FTMS method for the bracketing of less acidic and less basic sites in molecules that have multiple acidic and basic sites; the experimental procedure and limitations have been described previously.[8,9,12,13] In this setup, nucleobase ions produced after reaction of the corresponding neutral with hydroxide ions are immediately removed from the first cell and transferred into the second cell. Reference acids are then injected into the second cell and allowed to react with the nucleobase ions. The first reaction cell is rich in neutral nucleobase concentration and over time, neutral-catalyzed isomerization leads to survival of only the most acidic ions. Transferring ions into the second cell immediately after their generation allows us to carry out the reaction between reference acids and nucleobase ions in the absence of neutral nucleobase. The same procedure can be applied to the bracketing of less basic sites as well.[10]

In our experiments, the ions are the reactants and the neutrals are in excess, creating pseudo-first-order conditions. Because the nucleobase is introduced via an external solids probe that, when introduced to the high vacuum, typically causes the pressure of the cell to rise to about 10^{-7} Torr, we utilize the following procedure (which has also been described previously) to ascertain the pressure of the neutral.[13] First, we obtain the pseudo-first-order rate constant for the reaction of hydroxide with the relevant neutral. We then assume that this reaction between hydroxide, which is very basic, and the neutral proceeds at the theoretical collisional rate.[74] We then use that calculated collisional rate constant to "back out" the neutral pressure. This procedure also precludes the errors which can arise from ion gauge issues (such as remote location and varying sensitivity for different substrates).[75]

For the proton affinity experiments, we also utilized the Cooks kinetic method in a quadrupole ion trap mass spectrometer.[76−79] This method involves the formation of a proton bound complex, or dimer, of the unknown (in our case, hypoxanthine) and a reference base of known proton affinity (eq 1, where "A" is hypoxanthine and "B_i" is a series of reference bases). Collision-induced dissociation (CID) of this dimer leads to the formation of either the protonated unknown or the protonated reference base. The ratio of these two protonated products yields the relative proton affinities of the two compounds of interest, assuming that the dissociation has no reverse activation energy barrier and that the dissociation transition structure is late and therefore indicative of the stability of the two protonated products. Both these assumptions are generally true for proton-bound systems.[79−81]

$$[AHB_i]^+ \xrightarrow{k_1} AH^+ + B_i \qquad [AHB_i]^+ \xrightarrow{k_2} B_iH^+ + A \tag{1}$$

$$\ln(k_1/k_2) = [PA(A)/RT_{eff})-\Delta(\Delta S)/R] - PA(B_i)/(RT_{eff}) \tag{2}$$

$$\ln(k_1/k_2) = \ln([AH^+]/[B_iH^+]) \tag{3}$$

$$GB^{app}(A)/RT_{eff} = PA(A)/(RT_{eff}) - \Delta(\Delta S)/R \tag{4}$$

For our proton affinity studies of hypoxanthine, we utilized four reference bases and measured the product ion distributions three separate times to ensure reproducibility. We also conducted the Cooks kinetic method experiments using the "extended" method.[82−86] This method has been well-described and involves acquiring ion abundance ratios at different collision energies (and therefore different effective temperatures (vide infra)), which allows for deconvolution of the enthalpic and entropic contributions. Equations 2−4 summarize the data analysis. Briefly, T_{eff} is the effective temperature of the dissociating proton bound complex in Kelvin. The term "$\Delta(\Delta S)$" is the difference in the ΔS associated with the two channels in eq 1. A plot of $\ln(k_1/k_2)$ (which is equal to $\ln([AH^+]/[B_iH^+])$, eq 3) versus $PA(B_i)$ yields the T_{eff} from the slope (eq 2) and the "$GB^{app}(A)$" from the intercept (eqs 2 and 4). Plotting eq 4 at different values of T_{eff} yields the proton affinity of hypoxanthine. We find that the standard deviation of our measurements is ± 3 kcal mol^{-1}.

For the kinetic method experiments, solutions of hypoxanthine and the reference bases were subjected to electrospray ionization (10^{-3} to 10^{-4} M solutions in methanol; a small amount of acetic acid is also added). The typical flow rate is 25 μL/min. An electrospray needle voltage of ~4500 V was used. The proton-bound complexes of hypoxanthine and the reference base were isolated and subjected to collision-induced dissociation (CID); the

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complexes were activated for about 30 ms. About 40 scans were averaged for product ions.

Throughout this paper, acidity is reported as ΔH_{acid} and is the change in enthalpy associated with deprotonation of a molecule HA to form H^+ and A^-. Proton affinity is reported as PA and is the negative change in enthalpy associated with protonation of a molecule B to form BH^+ (that is, protonation is exothermic but PAs are reported as positive values).[44] Gas-phase calculations were conducted at B3LYP/6-31+G* with Gaussian03.[87−92] All gas-phase structures, including those in the Supporting Information, were fully optimized; frequency calculations confirmed true minima, with no negative frequencies. Acidity and proton affinity values are reported as ΔH at 298 K, which allows for direct comparison with the experimentally measured values. Solvation studies were conducted using the CPCM-SCRF method (full optimizations at B3LYP/6-31+G*; UAKS cavity) as implemented in Gaussian03.[93,94] This method has been shown to be reliable for neutral and ionic organic molecules.[95] A dielectric constant of 78.4 was used in order to simulate an aqueous environment; we did not conduct frequency calculations so we report ΔE values.

Acknowledgment. We thank the National Science Foundation (CHE-0092215 and CHE-0543038), the Alfred P. Sloan Foundation, and the Rutgers Busch Grant Program for support, and the National Center for Supercomputing Applications, the High Performance Computing Center at the University of Kentucky, and the Boston University Scientific Computing Facilities for computational resources. We are also grateful to Dr. Richard Tschirret-Guth of Merck Research Laboratories for effecting the donation of the Finnigan-LCQ quadrupole ion trap mass spectrometer used herein.

Supporting Information Available: Cartesian coordinates for all calculated species discussed herein (hypoxanthine, adenine, and guanine), as well as all tautomers of hypoxanthine and the acidities and proton affinities of each. This material is available free of charge via the Internet at http://pubs.acs.org.

JO070996X

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