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Computational Studies of the Gas-Phase Thermochemical Properties of Modified Nucleobases

Mu Chen and Jeehiun K. Lee*

Department of Chemistry and Chemical Biology Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08901, United States

ABSTRACT: In this Synopsis, we highlight some recent computational studies of the gas-phase thermochemical properties of modified nucleobases. Although this field is relatively nascent, we aim herein to show a few examples of insights that have already been gained by gas-phase calculations. We focus on modified nucleobases that are substrates for enzymes that excise damaged bases from DNA. Because these enzymes have hydrophobic active sites, calculations in the "ultimate" nonpolar environment of the gas phase prove to be particularly relevant, providing insight into enzyme mechanism.

Genomes are defined by the four DNA bases adenine (A), guanine (G), thymine (T), and cytosine (C). In addition to these major players, however, are a host of related species—modified nucleobases. Nucleobase analogues can play roles both innocent and guilty: some are naturally occurring and are found in various biosynthetic pathways, while others arise from damage events and are deleterious to the genome.[1,2] Still others are manmade, synthesized for the purpose of better understanding biological mechanisms that involve the normal, healthy counterparts.

In this Synopsis, we discuss some recent insights gained through the computational study of the gas-phase thermochemical properties of modified nucleobases. The study of the gas-phase properties of nucleobases is relatively recent, and a limited number of studies on *modified* nucleobases have been conducted.[3−22] Although both gas-phase experimental as well as calculational methodology have been applied to this area, in this paper we focus on computational work. To highlight the utility of such studies, we concentrate on substrates for two enzymes involved in DNA repair: thymine DNA glycosylase and MutY.[1,2]

Thymine DNA Glycosylase. *Gas-Phase Acidity Calculations Used To Model Reactivity in Enzyme Active Sites.* Nucleobases are chemically reactive and are modified by both endogenous and exogenous sources.[1,2] Organisms have developed a "base excision repair" (BER) pathway that counteracts such damage.[1,2] The leading edge of BER are DNA glycosylase enzymes, which excise the damaged base via scission of the C1′(sugar)−N (N1 for pyrimidine and N9 for purine nucleobase) bond (Figure 1). Such enzymes are impressive in their specificity, targeting damaged bases that often differ minimally from their normal counterparts and which exist in a vast excess of undamaged DNA.

Because glycosylases cleave the C1′−nucleobase N bond (Figure 1), the acidity at the nucleobase nitrogen position (deprotonation of N−H) of the free nucleobases can be used to reflect the leaving group ability of the corresponding deprotonated nucleobase (Figure 1).[3,12] The more acidic the nucleobase nitrogen position, then presumably, the better the

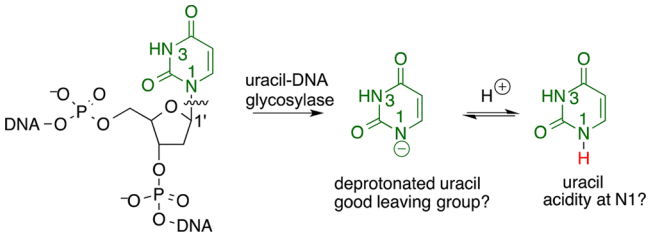

Figure 1. Glycosylases cleave the C1′−N bond in nucleotides. If the nucleobase is not protonated prior to cleavage, the nucleobase is excised in deprotonated form, as is shown here for uracil.

deprotonated nucleobase is as a leaving group and the more prone the corresponding glycosidic bond is to heterolytic cleavage. This was first proposed by our group for uracil (Figure 1) in the context of an enzyme that cleaves uracil from DNA, uracil DNA glycosylase (UDGase).[3] We further postulated that the *gas phase* acidity, as opposed to the *aqueous* acidity, might be of more relevance to the enzymatic cleavage because enzyme active sites can be quite hydrophobic.[23−30] We in fact did find that the calculated gas phase acidity of uracil is surprisingly high, compared to its relative acidity in water. While the N1−H of uracil has a pK_a of roughly 10, the gas-phase acidity of that position is calculated to be 331 kcal/mol (ΔG_{acid}). Thus, while the aqueous pK_a of the uracil N1−H is comparable to that of ammonium, the gas-phase acidity is comparable to that of hydrochloric acid! This surprisingly high acidity implies the natural predisposition of the glycosidic bond in uracil deoxyribose monophosphate to scission. Although in water deprotonated uracil is not a particularly good leaving group, our studies showed that in a nonpolar environment, relatively speaking, it should be comparable to chloride in leaving group ability—quite good. Subsequent to this proposal that the enzyme provides a hydrophobic site and that deprotonated uracil is a viable leaving group under such conditions, experimental studies

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with uracil DNA glycosylase revealed that, indeed, the deprotonated uracil anion (as opposed to a preprotonated substrate) is in fact the leaving group.[31−33]

This early work established that gas-phase acidity calculations could be of relevance to enzyme mechanism by providing information on leaving group ability in a nonpolar environment. This idea was expanded upon nicely by Drohat and co-workers, who examined a different glycosylase, human thymine DNA glycosylase.[34]

While uracil DNA glycosylase evolved to remove uracil, and only uracil, from DNA, there are other glycosylases which possess "broad specificity", meaning they cleave a wide range of damaged bases ("broad") while leaving undamaged bases untouched ("specificity"). Human thymine DNA glycosylase (hTDG) is one such glycosylase, excising thymine from G·T mispairs, as well as 5-bromouracil, 5-formyluracil, 5-hydroxyuracil, 5-hydroxymethyluracil, 3,N^4-ethenocytosine, 5-hydroxycytosine, thymine glycol, and hypoxanthine (with a preference for bases that are paired with guanine).[35] However, hTDG is also specific in that it does *not* excise cytosine. This specificity is remarkable in light of the fact that there are many more normal G·C pairs than there are G·T or other mispairs, yet hTDG does not remove the more prevalent cytosine.

Drohat and co-workers postulated that the hTDG specificity might arise from *N*-glycosidic bond stability, where damaged bases might be more prone to scission (better leaving groups) than the normal undamaged bases.[34] In addition, the active site could further contribute to that specificity by providing a nonpolar environment that might enhance the differences in leaving group ability. To probe this possibility, Drohat and co-workers calculated the gas-phase acidity at the N1 position for a variety of hTDG substrates (Figure 2).

The substrates in Figure 2 are organized by group (uracil versus cytosine analogues); within the group, they are ordered by acidity (least to most acidic, which is equivalent to decreasing

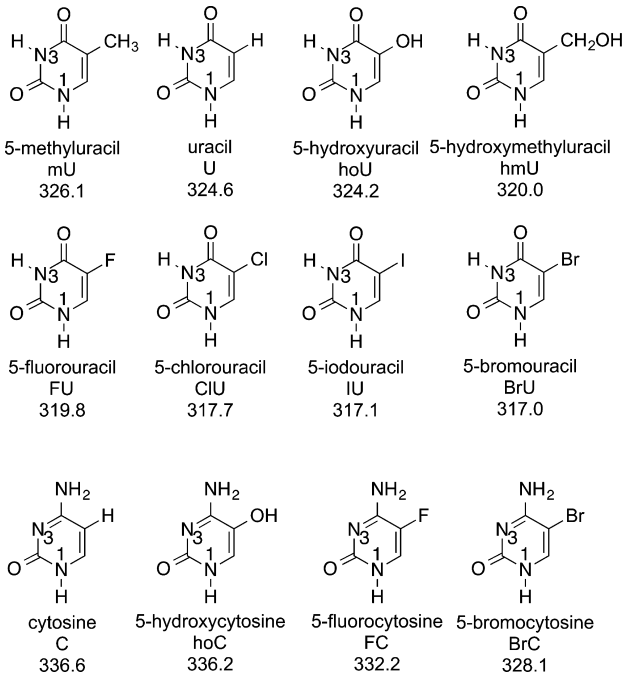

Figure 2. Gas-phase acidities of uracil, cytosine, and derivatives (ΔG_{acid}, kcal/mol, 298 K), calculated at MP2(full)/6-311+G(2d,2p)//MP2-(full)/6-31G(d). Data from ref 34.

ΔG_{acid} values). As would be expected, electron-donating groups such as methyl decrease acidity, whereas electron-withdrawing groups such as the halogens increase acidity. Overall, the uracil derivatives are more acidic than the cytosine. A plot of $2.3RT\log(k_{max})$ for hTDG-catalyzed excision versus ΔG_{acid} is linear, indicating that hTDG activity is highly correlated to N1 acidity of the target base. Drohat and co-workers also found a linear relationship between $\log(k_{max})$ and pK_a at N1 in aqueous solution. This reflects a high correlation between the gas- and solution-phase acidities for these particular nucleobases. More important, they found that the differences in N1 acidities are almost *4-fold greater* in the gas phase than in aqueous solution. Such a result indicates that acidity differences among nucleobases is enhanced in a hydrophobic active site, providing a means for discriminating the normal from the damaged bases.

A last point to consider is that although a hydrophobic site will enhance differences in leaving group ability for damaged versus undamaged bases, the reaction may overall be slower since charge development becomes less favorable. Thus, the specificity gained by increasing differences in leaving group ability might be counterbalanced by overall more sluggish cleavage. Drohat and co-workers noted that structural studies do point to backbone amides and water molecules which could provide hydrogen-bonding interactions to substrates in the hTDG-active site.[34,36] Furthermore, calculations by Wetmore and co-workers show that gas-phase acidities of uracil and derivatives are increased by hydrogen bonding.[18,19] Specifically, using DFT methods, they found that hydrogen-bonding interactions could increase the acidity of uracil derivatives by up to 12 kcal/mol. The hydrophobic environment can therefore render the damaged bases, relatively speaking, better leaving groups, while specific hydrogen-bonding interactions can accelerate the overall process.

Our studies of uracil and the subsequent study of hTDG by Drohat et al. established that gas phase acidity calculations can be relevant for biological mechanistic insight. Calculations showed that the nonpolar environment of hTDG will aid in the discrimination between normal and damaged bases, because the differences in acidity between these is much greater in a hydrophobic environment. This application of gas phase acidity calculations has since found utility with other glycosylases that display broad specificity.[8−11,14]

Gas-Phase Acidity Calculations and Cytosine Demethylation. Cytosine methylated at the 5-position is both biologically useful and harmful. 5-Methylcytosine (mC) is an epigenetic mark that influences biological processes, including transcription and development.[37] It can also be produced aberrantly, in which case it is a damaged base that requires removal. Only recently was a biological pathway to convert mC back to cytosine established, when it was found that a family of dioxygenases (ten-eleven translocation, or TET) can oxidize mC to 5-hydroxymethylcytosine (hmC) and 5-formylcytosine (fC) (Figure 3).[38,39] TET enzymes can further oxidize fC to 5-carboxylatecytosine (caC).[40−42] No mammalian glycosylase has been found that can excise hmC, but hTDG removes fC and caC (with the former being a much faster process).[41,43−48] In 2012 and 2013, two groups used gas-phase acidity calculations to probe the cleavage of fC and caC by hTDG, in the context of cytosine demethylation.[15,17]

Using DFT methods, Williams and Wang calculated the gas phase acidities of a series of 5-substituted cytosine derivatives (mC, hmC, FC, BrC, fC, and ca(H)C (where the carboxylate is protonated)), as well as of thymine (T).[17] They found that T, fC,

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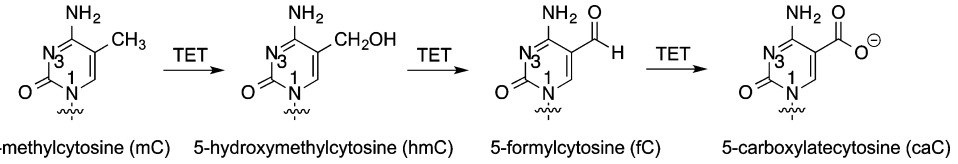

Figure 3. Oxidations catalyzed by TET.

and ca(H)C were more acidic at N1 than the other 5-substituted cytosine derivatives (acidities for these three most acidic substrates shown in Figure 4). This is consistent with the fact

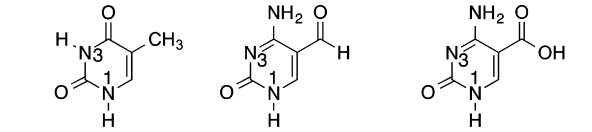

Figure 4. Gas-phase acidities of thymine, 5-formylcytosine, and neutral 5-carboxylcytosine (ΔG_{acid}, kcal/mol, 298 K), calculated at B3LYP/6-31+G(d).[49] Also listed is k_{max} for cleavage by hTDG (22 °C, min^{-1}) for thymine, 5-formylcytosine, and neutral 5-carboxylcytosine. Data from refs 17 and 50.

that these three substrates are also cleaved more quickly by hTDG than the other derivatives. Although the rate constants for cleavage do not track precisely with acidity (ca(H)C is more acidic than thymine and yet has a lower rate constant for cleavage, Figure 4, k_{max} in min^{-1}), the authors' point is that, overall, these three substrates are most acidic and are also cleaved more quickly by hTDG than other derivatives. Williams and Wang also calculated the exothermicities and barriers for *N*-glycosidic bond cleavage for the corresponding nucleotides and found that these values also correlate to the N1 acidities. Thus, the N1 acidities do reflect propensity for cleavage, consistent with the prior Drohat study.[34] The results are also consistent with a mechanism of cytosine demethylation involving the TET-catalyzed transformation of mC to fC and/or ca(H)C (Figure 3), followed by hTDG excision.

Lee and Drohat also carried out gas-phase calculations toward understanding cytosine demethylation and hTDG's role.[15] While Williams and Wang calculated the acidity of the neutral ca(H)C substrate; Lee and Drohat focused on the deprotonated anion, caC, which is the form expected to predominate under physiological conditions.

For fC, we calculated a very high gas-phase acidity (ΔG_{acid} = 320 kcal/mol, Figure 5). This acidity, as was also found by Williams and Wang, is significantly higher than that of other hTDG substrates. This high acidity is attributable to both the electronic and resonance stabilization properties of the formyl.[51] Williams and Wang had noted that fC is much more acidic than FC (Figure 5), despite both groups having similar Hammett inductive σ values (0.35 for CHO versus 0.34 for F). However, the formyl group can further enhance acidity through resonance, so the σ^- value (1.03 for CHO), which accounts for resonance, is relevant.[51,52] The importance of this resonance is reflected in the corresponding acidities (320.2 for fC and 331.6 for FC).

Although fC is very acidic and therefore presumably very cleavable, the caC anion is calculated to be not at all acidic (Figure 5), due to the formation of a dianion in the gas phase. We found, however, that protonating caC at N3 increases the

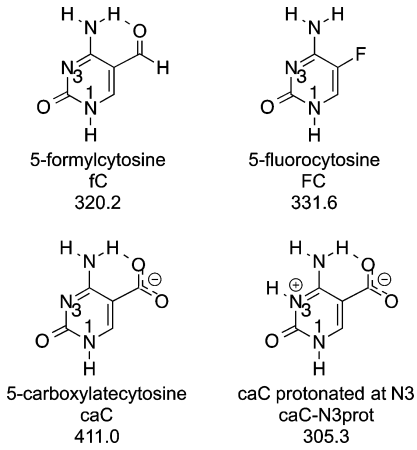

Figure 5. Calculated gas-phase acidities (ΔG_{acid}, kcal/mol, 298 K) for formyl, 5-fluoro, 5-carboxylate, and N3-protonated 5-carboxylatecytosine at B3LYP/6-31+G(d).

calculated gas-phase acidity by more than 100 kcal/mol (Figure 5). Since protonation at N3 increases N1 acidity so drastically, we proposed that acid catalysis (enzymatic protonation of N3) could be an effective strategy for the excision of caC by hTDG. The fact that caC is excised roughly 5-fold more slowly than fC is explained by the probable presence of a low population of N3-protonated caC and a higher population of the cleavage-resistant anion caC. Mutational analysis by Lee and Drohat and coworkers lent further experimental evidence to support these hypotheses.

These gas-phase acidity studies by Williams and Wang and Drohat and Lee established that fC is very acidic and should therefore be easily cleaved by hTDG; as long as TET can convert mC to fC (Figure 3), then hTDG can "take care" of the mutation. caC must be in neutral form for efficient cleavage; as the carboxylate anion predominates at physiological pH, hTDG may provide acid catalysis or hydrogen bonding to N3 to improve excision rates.

In all these reported studies of hTDG, gas-phase acidity calculations, which are relatively straightforward to execute, provide an insightful and simple means of assessing enzyme mechanism. Acidity measurements in water are more difficult to execute, and potentially less relevant for nonpolar active sites. This work shows the power of gas phase acidity calculations of modified nucleobases to lend insight into the enzymes that act upon these substrates.

MutY: Gas-Phase Acidity Calculations of Synthetic Adenine Analogues To Vett Possible MutY Mechanisms. While there are many modified nucleobases that arise biologically (good and bad), there are also those which are modified by scientists for the purpose of understanding biological mechanisms.

MutY is a glycosylase that does not target a damaged base but instead cleaves a normal base that is hydrogen bonded to a damaged base. One of the most common lesions found in DNA

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is 8-oxo-7,8-dihydroguanine (OG).[53−55] During DNA replication, if an OG is present, an adenine will be inserted opposite the OG to form a relatively stable OG:A mismatch.[56] This is considered a mismatch since undamaged guanine prefers to bind to cytosine, not adenine. MutY catalyzes the removal of adenine from OG:A mispairs in DNA.[57−59] This makes MutY a particularly intriguing enzyme as it is able to differentiate an A paired to OG from an A paired to a thymine.

The David group has conducted extensive analysis of the MutY mechanism. One key approach has been the use of synthetic adenine analogues that are designed to test which structural features of adenine are important for recognition and removal.[60−63] In a recent collaborative study, our and the David groups used computational methods to calculate the properties of synthetic adenine analogues, comparing the results to experimental data to lend insight into the MutY mechanism.[13]

Adenine and analogues are shown in Figure 6. 7-Deazaadenine (Z), 3-deazaadenine (Z3), and 1-deazaadenine (Z1) are missing nitrogen at the N7, N3, and N1 positions, respectively (relative to adenine). These three analogues were designed to test the importance of the nitrogen for binding and activity in MutY.[60,62,63] The nonpolar isosteres Q, M, and B were designed to test how important the polarity of adenine is for recognition and repair.[61,62]

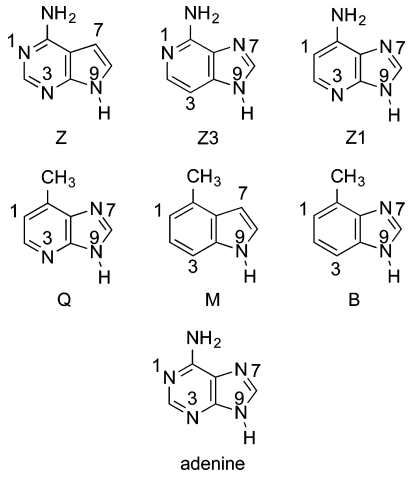

Figure 6. Adenine and synthetic analogues studied by Lee, David, and co-workers, ref 13.

For the MutY-catalyzed reaction, our goal was to ascertain whether a correlation exists between acidity of the synthetic analogues (at the N9 position) and their propensity for MutY cleavage. Two mechanisms were initially considered (Figure 7). For mechanism A, the gas-phase acidities at N9 for the adenine analogues were calculated, resulting in an acidity trend of A > Z3 > Q > B > Z1 > Z > M (where A is most acidic). This differs quite markedly from the trend for MutY cleavage: A > Q > Z1 > Z3 > B ≫ Z, M. Because these two trends do not correlate, preprotonation to enhance leaving group ability (Figure 7, mechanism B) was considered. The acidity that would correlate to this would be the acidity of the N7-protonated substrates. N7-protonation was found to greatly increase the N9−H acidity, by more than 100 kcal/mol, but the acidity trend still does not correlate to the MutY excision trend. We thus modeled the effect of a hydrogen bond at N3 on the N9 acidity, as experimental evidence indicated the importance of N3.[64−67] Using HF as a simple hydrogen bond donor, we recalculated the N9 acidities (Figure 8). The hydrogen bond at N3 increases acidity of the N7-protonated substrates at N9 by about 2−3 kcal/mol. The resultant acidity trend is A > Z3 ∼ Q > Z1 > B ≫ Z, M. This trend compares quite well to that of MutY excision (A > Q > Z1 > Z3 > B ≫ Z, M). The only substrate that appears out of place is Z3. The gas-phase acidity would imply highly facile cleavage by MutY, which is not experimentally observed. David and co-workers postulated it could be that the lack of N3 renders N7

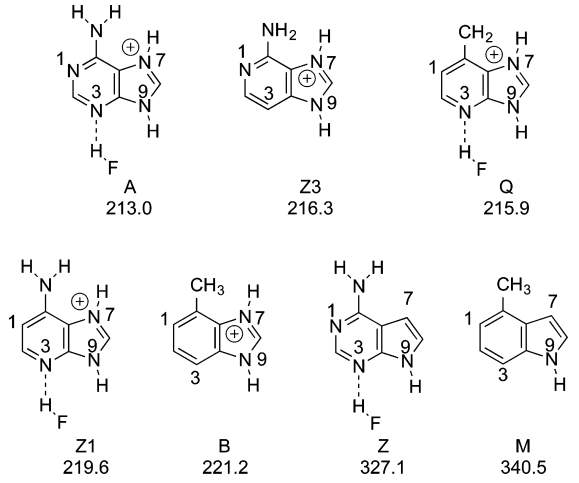

Figure 8. Calculated N9 acidities for adenine and analogues protonated at N7 and hydrogen bonded to HF at N3 (ΔG_{acid}, B3LYP/6-31+G(d), kcal/mol)).

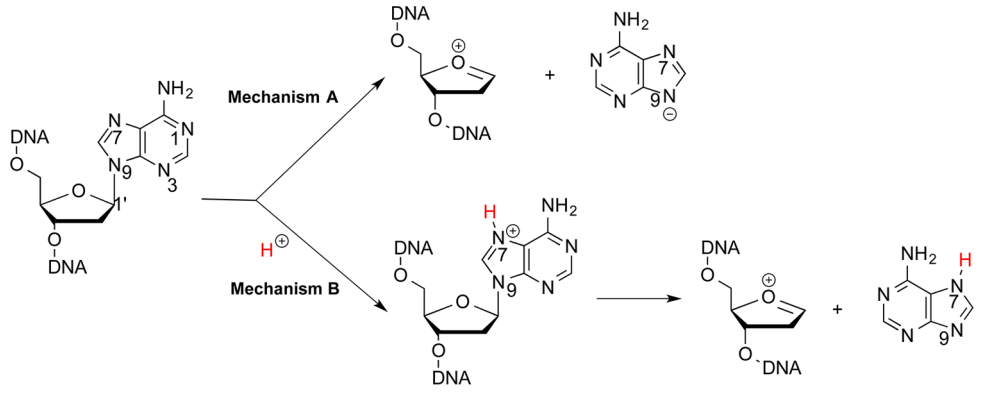

Figure 7. Two possible mechanisms of MutY cleavage.

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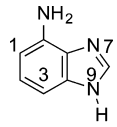
more difficult to protonate, thus slowing down excision; gas-phase proton affinity calculations support this hypothesis.[13]

MutY thus provides a nice example of how experimental enzymatic data on synthetic analogues can be used in conjunction with gas-phase acidity calculations to reveal mechanism. Calculated N9 gas-phase acidities of these analogues did not correlate to MutY excision rates, indicating that simple scission is not the operative enzyme mechanism. Next, preprotonation at N7 by the enzyme was considered. The gas-phase acidity model for this is the N9 acidity of the N7-protonated substrates. Still, the N9 acidities and MutY excision rates did not correlate. We then calculated the N9 acidity of N7-protonated structures with hydrogen bonding at N3. These acidities largely correlated with the MutY excision rates, thus indicating a viable mechanism for MutY: preprotonation at N7 with hydrogen bonding at N3. Current enzyme experimental work is consistent with this proposal.[13,57,62,63,68,69]

This work also resulted in a prediction, which has yet to be tested. Adenine analogue Z13 (Figure 9) is missing an N at both



Z13

Figure 9. Adenine analogue Z13.

the 1 and 3 positions and represents a logical next step in synthetic substrate design. Acidity calculations predict that MutY should excise Z13 very slowly, even more slowly than B (although more efficiently than Z and M). In contrast, for the nonenzyme-catalyzed reaction, acidity calculations in a water dielectric predict that Z13 should be cleaved more quickly than adenine.

Conclusions. The computational study of the gas-phase thermochemical properties of modified nucleobases is relatively nascent. In this Synopsis, we give a taste for what potential insights into biological mechanism gas-phase calculations of modified nucleobases can yield. The reported studies of hTDG show that gas-phase calculations are relevant to mechanisms that occur in hydrophobic environments, including enzymes. Gas-phase acidity calculations are relatively straightforward to execute yet correlate startlingly well to enzyme data. Although work to date on modified nucleobases has focused on acidity as related to enzymes that cleave these damaged bases, further gas-phase calculations will surely be of importance for any mechanism involving a hydrophobic environment.

The study of MutY highlighted another role for gas-phase acidity calculations of modified nucleobases. Many enzyme studies involve the synthesis and examination of unnatural analogues that are designed to test specific features of the enzyme active site. In the case of MutY, acidity calculations of such analogues provided a simple but quite accurate means of assessing the likelihood of various mechanisms, through correlation of acidity with enzyme activity. Although to date MutY is the only example of this application, future computational studies of other synthetic analogues for other enzymes could yield similar insights.

These examples herein show how relatively simple gas-phase acidity calculations can yield tremendous insights into enzyme activity. The field is young and we expect such calculations to continue to play a role in mechanistic investigation and provide predictions which can then be tested by enzymologists, forming a complementary relationship between gas-phase calculations and enzyme work.

AUTHOR INFORMATION

Corresponding Author

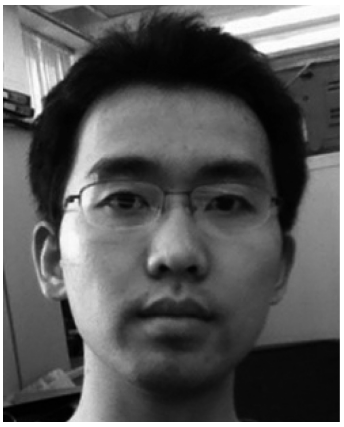
*E-mail: jee.lee@rutgers.edu.

Notes

The authors declare no competing financial interest.

Biographies

Jeehiun Katherine Lee received her B.A. at Cornell University and Ph.D. at Harvard University. After completing postdoctoral work with Professor Kendall Houk at UCLA, she moved to Rutgers University, where she is now Professor of Chemistry.



Mu Chen recently obtained his Ph.D. degree in Dr. Jeehiun K. Lee's research group at Rutgers University, the State University of New Jersey. His Ph.D. research focused on the gas-phase studies of organic reactivity using computational and mass spectrometric methods. He is now a Senior Scientist at Frontage Laboratories, Exton, PA, where he develops LC−MS methods for bioanalysis.

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