ACIDITY AND PROTON AFFINITY MEASUREMENT OF CYTOSINE BY MULTIPLE METHODS IN THE GAS PHASE

by

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ABSTRACT OF THE THESIS

Acidity and Proton Affinity Measurement of Cytosine by Multiple Methods in the Gas Phase

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The measurement of the intrinsic acidity of nucleic bases is essential for understanding the fundamental properties in biological systems. The Hydrogen bonding is critical to DNA stability and reactivity of oligonucleotides. The strength of hydrogen bonding can be gauged by the intrinsic acidity of the donor NH groups and the intrinsic basicity of acceptor atoms. Acidity is also indicative of the leaving group ability of a nucleobase in deglycosylation processes. In this dissertation, we examine the gas phase acidity and proton affinity of cytosine using Fourior transform mass spectrometry (FTMS), liquid chromatography mass spectrometry (LCMS) and ab initio calculations at B3LYP/6-31+G*. The experimental gas phase acidities and proton affinities were established using bracketing method, equilibrium method and Cooks kinetic method. Finally, we discuss the tautomer problem and deuterated experiments and the possible mechanism of the base excision repair by TDG enzyme.

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Chapter 1

Introduction

1.1 Acidity and proton affinity

Measuring the acidity and proton affinity of nucleobases is of essential importance in many biological systems. The first reason is that the NH-O and NH-N hydrogen bonds that hold base pairs together are dependent on the intrinsic acidity of the donor NH groups and the proton affinity of the acceptor atoms.[1-4] Secondly, understanding the intrinsic reactivity of nucleobases can shed light on some of the BER (base excision repair) mechanisms for which those nucleobases are excised by DNA glycosidase.[5, 6]

1.2 Gas phase

The gas phase is a valuable environment to mimic the environment of biological systems and allows us to establish the properties and reactivity of biological molecules, since recent studies indicate that many environments in biosystems, especially the interior of proteins and DNA, are nonpolar.[7-9] Also, the comparison of acidity and proton affinity studies in the gas phase with those in the condensed phase can further our understanding of how solvent influences nucleobase reactivity. Lastly, gas phase studies provide a valuable link between theoretical calculation and experimental data.[7, 10]

1.3 Cytosine

Cytosine is one of the four main nucleobases in DNA and is paired with guanine. It was first discovered in 1894 when it was isolated from calf thymus tissues.[11] From previous studies, it is known that cytosine exists predominantly as the C1 form (Figure 1) in solution and in the solid state.[12, 13] However, in the gas phase, calculations and some experiments show that cytosine is

found to coexist with its tautomers (Figure 1).[14-17]

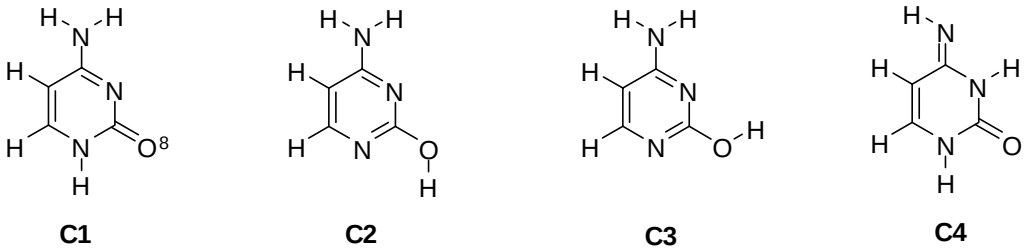


Figure 1.Tautomers of cytosine

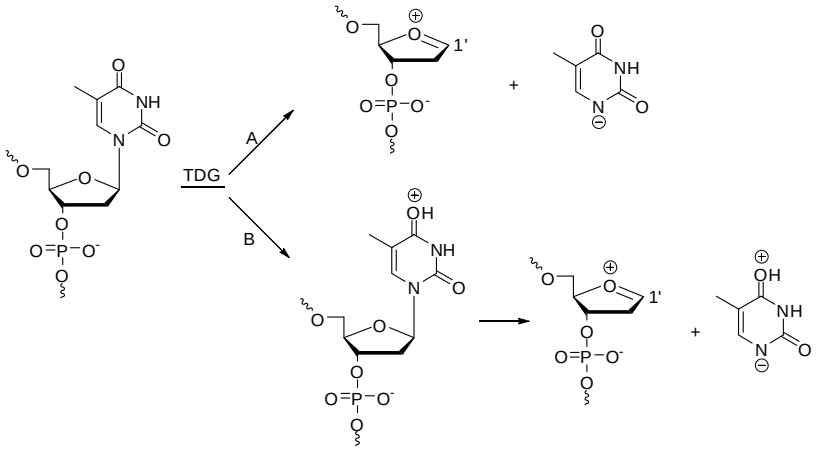
In this paper, several experimental methods are used to determine the gas phase acidity and proton affinity and, we hope, to shed light on what tautomers are present under our experimental conditions.

1.4 Thymine-DNA Glycosylase

TDG (Thymine-DNA Glycosylase) is an enzyme in human cells that excises a broad range of bases.[18] It excises thymine from G-T mispairs and also removes many other lesions, with a strong preference for bases (such as thymine glycol, hypoxanthine, 5-hydroxy-C, 5-bromo-U, 5-formyl-U, 5-hydroxy-U, 5-hydroxymethyl-U, 5-chloro-U, 5-iodo-U, 5-fluoro-C, 5-bromo-C, T (5-methyl-U) and 3,N^4-etheno-C) that are paired with guanine and located at CpG sites, but avoids excising cytosine from a normal G*C pair.[18-23] Because there is no obvious handle as to how cytosine is differentiable from the other substrates, the mechanism is of much interest. Two possible mechanisms could be proposed for the base excision repair by TDG: one where the base leaves as a deprotonated anion (Scheme 1A); the other where the base is protonated first and leaves as a neutral (Scheme 1B). Drohat and coworkers studied the relationship between the solution phase acidity of a series of nucleobase substrates and the kinetics of these substrates cleaved by TDG in solution. Their studies indicate that the nucleobase is cleaved in its deprotonated form (the more

acidic the nucleobase is, the easier the excision is).[18] Since the active site of the enzyme TDG is solvent free, we could study the reactivity of the normal and some damaged nucleobases in the gas phase to reveal the mechanism of this biological process. In our group, we have measured the reactivity of cytosine, and two damaged nucleobases, hypoxanthine and thymine, which could also be cleaved by TDG. We can then compare the gas-phase acidity and proton affinity of cytosine, thymine and hypoxanthine in this paper to study the pathway that the TDG BER (base excision repair) could undergo.

The gas-phase acidity of cytosine at N1 should correlate to the leaving group ability of the cytosine N1 anion in a nonpolar active site of TDG. If cytosine is less acidic than other substrates that could be cleaved by TDG, the excision of mismatched nucleobases would favor pathway A in which the base leaves as an anion. If cytosine is less basic than other substrates that could be cleaved by TDG, the excision of mismatched nucleobases could undergo pathway B, since the protonation step facilitates the cleavage of the neutral nucleobase.



Scheme 1

Chapter 2

Methodology

2.1 Computation.

The gas phase stability of different cytosine tautomers, as well as the gas phase acidity and proton affinity of multiple sites of the cytosine tautomers were calculated using Gaussian03, a quantum mechanical software package.[24] In the gas phase, acidity is defined as the enthalpy change associated with the gas-phase deprotonation of a molecule HA to form H^+ and A^-. Proton affinity is defined as the negative enthalpy change associated with the gas phase protonation of a molecule B to form BH^+. The geometries of the cytosine tautomers and their corresponding protonated and deprotonated forms were fully optimized by the density functional three-parameter model (B3LYP) at 6-31+G*. Frequency calculations were also carried out using the same level and method. Acidity and proton affinity values are reported as ΔH at 298 K.

2.2 Experiment.

All chemicals are commercially available and were used as received. The bracketing and equilibrium experiments were conducted on a dual-cell Finnigan 2001 Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR-MS or FTMS).[25-28] The dual cell, which sits in the center of the chamber coaxially with the magnetic field produced by a 3.3 T superconducting magnet, is the core component of the FTMS. It consists of two adjoining 2 in. cubic cells that are pumped to a baseline pressure of less than 1×10^{-9} Torr. The left side of the dual cell is called the source cell, and the right side of the dual cell is called the analyzer cell. Each cubic cell contains 3 pairs of plates: trapping plates, excitation plates and detection plates.[25] A heated batch inlet system

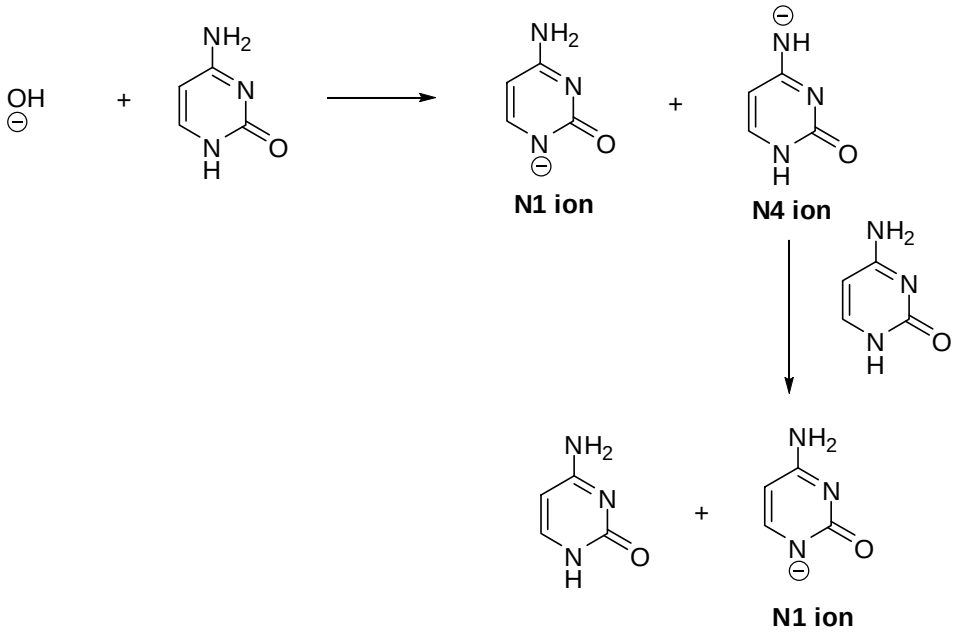
or a heated solid probe is used to introduce neutral samples into the FTMS. A trapping potential of 2 V (positive if positive ions are being trapped and negative if negative ions are being trapped) is applied to the trapping plates which are perpendicular to the magnetic field at all times except when ions are being transferred from one cell to another. Ion transfer is accomplished by temporarily grounding (40-150 μs) the trapping plate that separates the two cells. Ions can be transferred from one cell to another through a 2 mm hole in the center of the trapping plate. Argon can be pulsed in to cool down the transferred ions, which raises the cell pressure to 10^{-5} Torr. Hydroxide ions and hydronium ions are generated by pulsing water into the FTMS cell and sending an electron beam through the center of the cell. The electron beam is 8 eV, 6 μA to generate hydroxide ions and is 20 eV, 6 μA to generate hydronium ions. The beam time is 0.5 s for both the generation of hydroxide and hydronium ions.

2.2.1 Experiment: Bracketing Method

To bracket the most acidic site, neutral cytosine is deprotonated by hydroxide ions to form cytosine ions in the source cell and is then transferred to the analyzer cell, which has a stable pressure of the neutral reference acid, to see whether or not there is proton transfer. Also, the reverse reaction is carried out to confirm the presence of proton transfer. That is, the reference acid is deprotonated by hydroxide ions to form the conjugate base in the analyzer cell and is then transferred to the source cell to react with neutral cytosine. Rapid proton transfer (i.e., near the collision rate) was taken as evidence that the reaction was exothermic and is indicated by a "+" in the summary tables of bracketing results.

Since cytosine has multiple acidic sites, the hydroxide ions can deprotonate all the sites in gas

phase (Scheme 2) due to the high proton affinity of hydroxide ions.[29] The less acidic sites can undergo isomerization to form the most acidic site in the presence of neutral cytosine. In bracketing the most acidic site, a two-second-delay time is given for the isomerization. The experimental setup for bracketing the most basic site is similar to the most acidic site (we generate the hydronium ions to protonate cytosine).



Scheme 2

In our experiments, the neutral molecules are in excess, creating pseudo-first-order conditions. The procedure to obtain the pressure of neutral, the pseudo-first-order rate constant and efficiency of the reaction have been described previously.[27, 30] Briefly, for acidity bracketing, we obtain the pseudo-first-order rate constant for the reaction of hydroxide with the neutral cytosine or neutral reference acid first. Then, we assume that this reaction proceeds at the theoretical collisional rate since hydroxide ion is very basic.[31] Next, the calculated collisional rate constant is used to "back calculate" the neutral pressure. For the proton transfer reactions between cytosine and reference

acid, the pseudo-first-order rate constant can also be obtained by plotting the ln (intensity of reactant ions) versus time (s). The slope of the linear plot is the rate constant of the reaction. The efficiency is the ratio of the pseudo-first-order rate constant and the calculated collisional rate constant.

For the most acidic site bracketing experiments, some of the results are single cell experiments (indicated by superscript “d” in Table 1). That is, both neutral compounds, cytosine and the reference acid, are injected into the same cell, usually the source cell, due to the high boiling point of the reference acid and limitations of the analyzer batch inlet (the temperature of source batch inlet can be controlled by the computer and can reach up to 423 K but the temperature of batch inlet on the analyzer side is controlled by the minitrol heater and can not reach to a temperature high enough to vaporize these reference acids). If the reference acids used in the single cell experiment have acidities close to cytosine, the proton transfer reactions are rather slow. When both neutral cytosine and reference acid are present in the same cell, the reverse reaction occurs, which would make the plot of ln (intensity of reactant ions) versus reaction time not a straight line. Then, the rate constant and efficiency of the proton transfer reaction cannot be obtained. Therefore, the "+" or “-” sign indicating the occurrence or absence of proton transfer is not given in Table 1. Equilibrium method 2 is used to measure acidity of cytosine by those reference acids with high boiling points, which is discussed in the equilibrium method section.

To bracket the less acidic site, it is necessary to transfer the ions to the neutral-cytosine-free cell (analyzer cell) immediately after generation (0.001s), because the less acidic sites can undergo isomerization to form the most acidic site in the presence of neutral cytosine (Scheme 3).[26-28] The experimental procedure and limitations have been described previously.[27, 28, 30]

2.2.2 Experiment: Equilibrium Method

The equilibrium method is an alternate method to obtain acidity and proton affinity by measuring the relative Gibbs free energy in the proton transfer reaction.[32-34] Reaction 1 is the proton transfer reaction setup in this method, in which HA represents neutral reference acid, and HC represents neutral cytosine. $\triangle G_{PT}$ of reaction 1 can be expressed by the difference of the acidity of HA and HC (Equation 2), which can also be obtained experimentally from the definition of the equilibrium constant K_{PT} (Equation 3). Therefore, the acidity of cytosine can be obtained since the acidity of the reference acid is known.

$$C^- + HA \underset{k_-}{\overset{k_+}{\rightleftharpoons}} A^- + HC \qquad (1)$$

$$\triangle G_{PT} = \triangle_{acidity}H_{(HA)} - \triangle_{acidity}H_{(HC)} = -RT \ln K_{PT} \qquad (2)$$

$$K_{PT} = \frac{k_+}{k_-} = \frac{[HC]\,[A^-]}{[HA]\,[C^-]} \qquad (3)$$

2.2.2.1 Experiment: Equilibrium Measurements, Method 1 (Deriving equilibrium from forward and reverse rate constants).

In bracketing experiments, the rate constants of the bimolecular ion-molecule reactions are measured. We can apply the rate constant k_+ and k_- obtained from bracketing experiments in this method to calculate the acidity of cytosine from Equation 2 and Equation 3.[34]

2.2.2.2 Experiment: Equilibrium measurements, Method 2 (Setting up an experimental equilibrium)

Both neutral cytosine and neutral reference acid are leaked into the source cell. Then cytosine

and reference acid ions are generated by hydronium or hydroxide ions. A Stored Waveform Inverse Fourier Transform (SWIFT) is applied to eject undesired ions out of the source cell. Only cytosine cations are left to react with neutral reference acid HA in the presence of neutral HA and HC. From the definition of the equilibrium constant (Eq. 3), the acidity of unknown can be obtained.[32]

2.2.3 Experiment: Cooks Kinetic Method

Cooks kinetic method is another method we use to obtain the acidity and proton affinity of cytosine. It is utilized in a quadrupole ion trap mass spectrometer, which has a quadrupole ion trap to analyze ions.[33-34] In this method, a proton bound complex of cytosine and a reference compound of known proton affinity or acidity first forms and CID (collision-induced dissociation) of the complex leads to the formation of either the cytosine ions or reference compound ions. The ratio of these two products yields the relative proton affinities or acidities of cytosine and the reference compound.

Let us take the measurement of proton affinity as an example. The proton bound complex of cytosine and reference base can be formed in solution. ESI (electrospray ionization) is used to vaporize the solution into the gas phase. Then, the desired proton bound complex of cytosine and reference base can be isolated by the mass spectrometer using DC and radio frequency (RF) oscillating AC electric fields. Then, CID (collision-induced dissociation) of the complex gives the protonated cytosine and protonated reference base (Equation 4). The ratio of these two products reflects the relative proton affinity of these two compounds (Equation 5). In the equations, B_i represents a series of reference bases with known proton affinity and A represents cytosine. T_{eff} is

the effective temperature of proton-bound dimer in Kelvin. T_{eff} can be obtained from the plot of ln ($[AH^+]$ / $[B_iH^+]$) versus the proton affinity of a series of reference bases. The slope of the plot is 1/ (RT_{eff}), in which R is the gas constant. The two assumptions of this method are: 1. The dissociation does not have a reverse activation barrier. 2. The difference of the entropy change ($\triangle_1^{\#}S$-$\triangle_2^{\#}S$) in equation 6 is negligible (the reference base has a similar structure to the unknown sample).[33-34]

$$[AHB_i]^{\oplus} \xrightarrow{k_1} B_iH^{\oplus} + A \qquad [AHB_i]^{\oplus} \xrightarrow{k_2} AH^{\oplus} + B_i \tag{4}$$

$$\ln\frac{[AH^+]}{[B_iH^+]} = \ln\frac{k_2}{k_1} = \frac{\triangle_1^{\#}G - \triangle_2^{\#}G}{RT_{eff}} = \frac{\triangle_1^{\#}H - \triangle_2^{\#}H}{RTeff} - \frac{\triangle_1^{\#}S - \triangle_2^{\#}S}{R}$$
$$\sim= \frac{1}{RT_{eff}}(PA_2 - PA_1) \tag{5}$$

For Cooks kinetic method experiments, solutions of cytosine and the reference bases (10^{-3} to10^{-4}M solutions in 4:1 water and methanol mixture; a small amount of acetic acid is added for positive mode and a small amount of ammonia for negative mode) were injected and vaporized by ESI. The typical flow rate was 25μL/min. The electrospray needle voltage was ~4500V. The proton-bound complexes were activated for about 30 ms, and about 40 scans were averaged for product ions.[30]

2.2.4 Experiment: Error Bar

The value of the bracketing results is the average of the larger value and smaller value of the

acidity or proton affinity including their error bar of the two reference compounds at the edge of the cutoff in the experiments. The error bar of the bracketing is the difference between the average and the larger value or smaller value of the acidity and proton affinity of the two reference compounds.[26-28]

The results of the acidity and proton affinity measurement from equilibrium method 1, equilibrium method 2 and the Cooks kinetic method are represented as the average of all the experimental results obtained in each method, respectively. The experimental average value in Table 10 is the average of the results from all the methods. The error bars in equilibrium experiments and the Cooks kinetic method are calculated following the equation below:

$(\text{Error Bar})^2 = \text{sum } \{(\text{Error bar of each reference compound})^2/(n-1) + (\text{standard deviation})^2\}$, where n is the number of the reference compounds used in each method.[35]

The error bar of the experimental average value in Table 10 is calculated similarly:

$(\text{Error Bar})^2 = \text{sum } \{(\text{Error bar from each experimental method})^2/(n-1) + (\text{standard deviation})^2\}$, where n is the number of methods used in the experiments.[35]

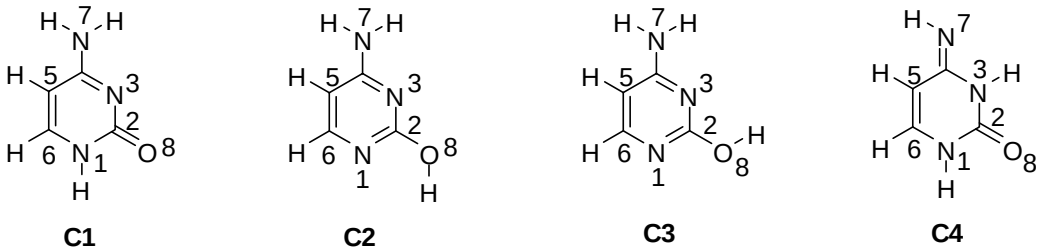
Chapter 3

Results

3.1 Computational Results

3.1.1 Tautomers

Among all the possible tautomers of cytosine, four of them are quite close in energy, and are the most stable structures (Figure 2). The remaining possible tautomers are less stable by at least 4.5 kcal mol^{-1}.[36] In addition to the canonical form (C1), there are two enol (C2 and C3) forms and one imino (C4) form. C2 is less stable than C1 by 1.7 kcal mol^{-1}; C3 is less stable than C1 by 2.5 kcal mol^{-1}; C4 is less stable than C1 by 2.2 kcal mol^{-1}. The order of the relative energies of these four tautomers is consistent with previous calculations that use density functional theory.[37]



C1 **C2** **C3** **C4**

0.0 kcal mol^{-1} 1.7 kcal mol^{-1} 2.5 kcal mol^{-1} 2.2 kcal mol^{-1}

Figure 2. Relative energies (ΔH) of the four most stable tautomers of cytosine, calculated at B3LYP/6-31+G, at 298 K.*

3.1.2 Acidity

The gas phase acidities for all the protons of cytosine calculated at B3LYP/6-31+G* are summarized in Figure 3.

The most acidic site of the C1 tautomer is the N1-H, at 343.3 kcal mol^{-1}. The two protons attached to N7 are slightly less acidic: the N7-H toward the C5 side is at 346.6 kcal mol^{-1} and the N7-H toward the N3 side is at 352.6 kcal mol^{-1}. The C-H protons are the least acidic: the C6-H calculates to 367.8 kcal mol^{-1} and the C5-H to 376.2 kcal mol^{-1}.

The most acidic site of the C2 tautomer is the O8-H, at 341.6 kcal mol^{-1}. The two protons attached to N7 are slightly less acidic: the N7-H toward the C5 side is at 349.9 kcal mol^{-1} and the N7-H toward the N3 side is at 354.1 kcal mol^{-1}. The C-H protons are the least acidic: the C5-H calculates to 383.4 kcal mol^{-1} and the C5-H to 391.1 kcal mol^{-1}.

The most acidic site of the C3 tautomer is the O8-H, at 340.8 kcal mol^{-1}. The two protons attached to N7 are slightly less acidic: the N7-H toward the C5 side is at 348.5 kcal mol^{-1} and the N7-H toward the N3 side is at 351.8 kcal mol^{-1}. The C-H protons are the least acidic: the C5-H calculates to 383.0 kcal mol^{-1} and the C5-H to 393.3 kcal mol^{-1}.

The most acidic site of the C4 tautomer is the N1-H, at 335.4 kcal mol^{-1}. The N3-H is slightly less acidic, at 350.4 kcal mol^{-1}. The C-H protons are the least acidic: the C6-H calculates to 366.7 kcal mol^{-1} and the C5-H to 376.1 kcal mol^{-1}.

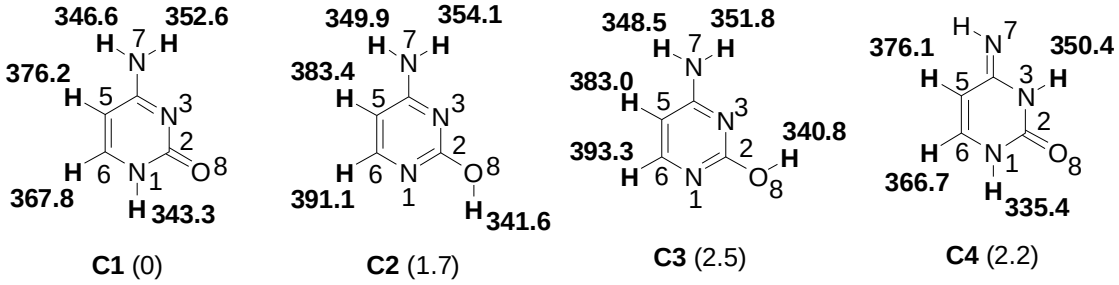


Figure 3. Calculated acidities (ΔH_{acid}) of the four most stable tautomers of cytosine at B3LYP/6-31+G, at 298 K. Parenthetical values are relative energies of the tautomers in kcal mol^{-1}. Values beside the acidic protons are acidities in kcal mol^{-1}. Acid protons are in bold.*

3.1.3 Proton Affinity

The gas phase proton affinities for cytosine tautomers at B3LYP/6-31+G* are summarized in Figure 4.[36]

The most basic site of the C1 tautomer is the N3 site, at 226.5 kcal mol^{-1}. The O8 site is slightly less basic, at 225.5 kcal mol^{-1}. The N7 site is the least basic, at 195.2 kcal mol^{-1}.

The most basic site of the C2 tautomer is the N3 site, at 218.8 kcal mol^{-1}. The N7 site is slightly less basic, at 197.0 kcal mol^{-1}. The O8 site is the least basic, at 180.1 kcal mol^{-1}.

The most basic site of the C3 tautomer is the N1 site, at 227.5 kcal mol^{-1}. The N3 site is slightly less basic, at 208.4 kcal mol^{-1}. The N7 site is the least basic, at 194.4 kcal mol^{-1}.

The most basic site of the C4 tautomer is the N7 site, at 228.8 kcal mol^{-1}. The O8 site is slightly less basic, at 198.8 kcal mol^{-1}. The N7 site is the least basic, at 182.7 kcal mol^{-1}.

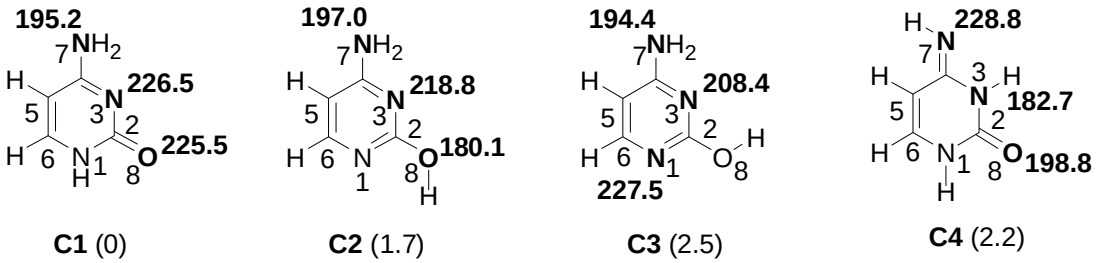


Figure 4. Calculated proton affinities (ΔH) of the four most stable tautomers of cytosine at B3LYP/6-31+G, at 298 K.[36] Parenthetical values are relative energies of the tautomers in kcal mol^{-1}. Values beside the basic sites are proton affinities in kcal mol^{-1}. Basic sites are in bold.*

3.2 Experimental Results

3.2.1 Acidity of the most acidic site

3.2.1.1 Bracketing method

For the most acidic site, the conjugate base of cytosine deprotonates methyl cyanoacetate ($\triangle H_{acid}$ = 340.8 ± 0.60 kcal mol^{-1}), but not 2, 4-pentanedione ($\triangle H_{acid}$ = 343.8 ± 2.1 kcal mol^{-1}); also 2, 4-pentanedione anion deprotonates cytosine but methyl cyanoacetate does not (Table 1). Therefore, the most acidic site of cytosine could be bracketed to be 343.1 ± 2.9 kcal mol^{-1}.

Table 1. Summary of results of acidity bracketing experiments for the most acidic site in cytosine

reference acid	name	$\triangle H_{acid}$[a] (kcal mol^{-1})	proton transfer[b]	
			ref. acid	conjugate base
CH_3COOH	acetic acid	348.1 ± 2.2	-	+
HCOOH	formic acid	345.3 ± 2.2	-[c]	+
$CH_3COCH_2COCH_3$	acetylacetone	343.8 ± 2.1	-[c]	+
CH_3OCH_2COOH[d]	methoxyacetic acid	341.9 ± 2.1	N/A[d]	N/A[d]
CH_2ClCH_2COOH[d]	3-chloropropionic acid	340.8 ± 2.7	N/A[d]	N/A[d]
CH_3OCOCH_2CN	methyl cyanoacetate	340.8 ± 0.6	+	-[c]
$C_7H_5F_3O$	m-cresol	339.3 ± 2.1	+	-
$CH_3CH_2ClCOOH$[d]	2-chloropropanoic acid	337.0 ± 2.1	+[d]	-[d]
Result		**343.1 ± 2.9**		

[a] Acidities are in kcal mol^{-1} and come from ref 38. [b] A "+" indicates the occurrence and a "-" denotes the absence of proton transfer. [c] The plot of ln(reactant ions) versus time is slightly curved due to the slow reaction and the leakage of the neutral from one cell to another. [d] The plot of ln (reactant ions) versus time is curved. Both neutral cytosine and neutral reference acids are injected into source cell --- single cell experiment.

3.2.1.2 Equilibrium method

Because it is difficult to find other reference acids with known acidities around 342 kcal mol^{-1} to narrow the error bar of bracketing results, we choose the equilibrium method to further measure the acidity of cytosine.

For the equilibrium measurements (method 1: deriving equilibrium from forward and reverse rate constants, see Experimental Section), the average acidity of cytosine is 340.9 ± 2.1 kcal mol^{-1} (Table 2).

Table 2. Summary of results of equilibrium method 1 for the most acidic site in cytosine

reference acid	name	$\triangle H_{acid}$[a] (kcal mol^{-1})	acidity of cytosine (kcal mol^{-1})
$CH_3COCH_2COCH_3$	acetylacetone	343.8 ± 2.1	340.8
CH_3OCOCH_2CN	methyl cyanoacetate	340.8 ± 0.6	341.1
m-CF_3-C_6H_4OH	m-cresol	339.3 ± 2.1	340.9
Average			**340.9 ± 2.1**

[a] Acidities are in kcal mol^{-1} and come from ref 38.

For the equilibrium measurements (method 2: setting up an experimental equilibrium, see Experimental Section), the average acidity of cytosine is 341.6 ± 2.3 kcal mol^{-1} (Table 3).

Table 3. Summary of results of equilibrium method 2 for the most acidic site in cytosine

reference acid	name	$\triangle H_{acid}$[a] (kcal mol^{-1})	acidity of cytosine
$CH_3(CH_2)_3COOH$	pentanoic acid	346.2 ± 2.1	342.9
HCOOH	formic acid	345.3 ± 2.2	341.7
$CH_3COCH_2COCH_3$	acetylacetone	343.8 ± 2.1	340.6
CH_3OCH_2COOH	methoxyacetic acid	341.9 ± 2.1	342.1
CH_3OCOCH_2CN	methyl cyanoacetate	340.8 ± 0.6	341.4
m-CF_3-C_6H_4OH	m-cresol	339.3 ± 2.1	340.8
Average			**341.6 ± 2.3**

[a] Acidities are in kcal mol^{-1} and come from ref 38.

3.2.1.3 Cooks kinetic method

The Cooks kinetic method is used to confirm the measurement of cytosine acidity of the most acidic site and proton affinity of most basic site. Especially for proton affinity measurement, the proton bound complex of two cytosine molecules or the proton bound complex of a cytosine and a reference base may show up after long reaction time in both bracketing and equilibrium experiments, which introduces complications and may lead to inaccurate results. Therefore, the Cooks kinetic method can be applied to confirm the previous results.

The average acidity of cytosine from Cooks measurements is 341.8 ± 2.1 kcal mol^{-1} (Table 4).

Table 4. Summary of results of Cooks kinetic experiments for the most acidic site in cytosine

reference acid	name	$\triangle H_{acid}$[a] (kcal mol^{-1})	acidity of cytosine (kcal mol^{-1})
CH_3OCH_2COOH	methoxyacetic acid	341.9 ± 2.1	341.8 ± 2.1

[a] Acidities are in kcal mol^{-1} and come from ref 38.

3.2.2 Proton Affinity of the most basic site

3.2.2.1 Bracketing method

For the most basic site, the conjugate acid of cytosine protonates 1-methylpyrrolidine $C_5H_{11}N$ (PA = 230.8 ± 2.0 kcal mol^{-1}), but not pyrrolidine C_4H_9N (PA = 226.6 ± 2.0 kcal mol^{-1}); also pyrrolidine $C_4H_{10}N^+$ cation protonates cytosine but 1-methylpyrrolidine cation $C_5H_{12}N^+$ does not. Therefore, the most basic site of cytosine is bracketed to be 228.7 ± 4.1 kcal mol^{-1} (Table 5).

Table 5. Summary of results of PA bracketing experiments for the most basic site in cytosine

reference base	name	proton affinity[a] (kcal mol^{-1})	proton transfer[b]	
			ref. acid	conjugate base
$C_6H_{13}N$	1-methylpiperidine	232.1	+	-
$C_5H_{11}N$	1-methylpyrrolidine	230.8	+	-
$C_5H_{11}N$	piperidine	228.0	+	+
C_4H_9N	pyrrolidine	226.6	-	+
C_6H_7N	3-picoline	225.5	-	+
C_5H_5N	pyridine	222.3	-	+
C_4H_9NO	n-methylpropionamide	220.0	-	+
Result		**228.7 ± 4.1**		

[a] Proton affinities are in kcal mol^{-1} and come from ref 38. [b] A "+" indicates the occurrence and a "-" denotes the absence of proton transfer.

3.2.2.2 Equilibrium method

For the equilibrium measurements (method 1: deriving equilibrium from forward and reverse rate constants, see Experimental Section), the average proton affinity of cytosine is 228.1 ± 3.0 kcal mol^{-1} (Table 6).

Table 6. Summary of results of equilibrium method 1 for the most basic site in cytosine

reference base	name	PA of ref. base[a] (kcal mol^{-1})	PA of cytosine (kcal mol^{-1})
$C_5H_{11}N$	1-methylpyrrolidine	230.8	230.8
$C_5H_{11}N$	piperidine	228.0	228.9
C_4H_9N	pyrrolidine	226.6	228.4
C_6H_7N	3-picoline	225.5	227.1
C_5H_5N	pyridine	222.3	225.5
Average			**228.1 ± 3.0**

[a] Proton affinities are in kcal mol^{-1} and come from ref 38.

For the equilibrium measurements (method 2: setting up an experimental equilibrium, see Experimental Section), the average proton affinity of cytosine is 227.7 ± 2.5 kcal mol^{-1} (Table 7).

Table 7. Summary of results of equilibrium method 2 for the most basic site in cytosine

reference base	name	PA of ref. base[a] (kcal mol^{-1})	PA of cytosine (kcal mol^{-1})
$C_5H_{11}N$	piperidine	228.0	228.2
C_4H_9N	pyrrolidine	226.6	227.5
C_6H_7N	3-picoline	225.5	227.3
Average			**227.7 ± 2.5**

[a] Proton affinities are in kcal mol^{-1} and come from ref 38.

3.2.2.3 Cooks kinetic method

The average PA of cytosine from Cooks measurements is 227.1± 2.2 kcal mol^{-1} (Table 8).

Table 8. Summary of results of Cooks experiments for the most basic site in cytosine

reference base	name	PA of ref. base[a] (kcal mol^{-1})	PA of cytosine (kcal mol^{-1})
$C_5H_{11}N$	1-methylpyrrolidine	230.8	227.3
$C_5H_{11}N$	piperidine	228.0	227.1
C_4H_9N	pyrrolidine	226.6	226.9
C_6H_7N	3-picoline	225.5	227.0
C_5H_5N	pyridine	222.3	227.2
$C_8H_{19}N$	1-octanamine	222.0	227.1
Average			**227.1 ± 2.2**

[a] Proton affinities are in kcal mol^{-1} and come from ref 38.

3.2.3 Acidity of Less Acidic Site

The conjugate base of cytosine deprotonates p-cresol ($\triangle H_{acid}$ = 350.3± 2.1 kcal mol^{-1}), but not 4-(trifluoromethyl)aniline ($\triangle H_{acid}$ = 353.3 ± 2.1 kcal mol^{-1}). Therefore, we bracket the acidity of less acidic site in cytosine to be 351.8 ± 3.6 kcal mol^{-1} (Table 9).

Table 9. Summary of results of bracketing of less acidic site in cytosine

reference acid	name	$\triangle H_{acid}$[a]	proton
C_5H_5N	pyrrole	359.4 ± 0.3	-
C_2H_2ClN	chloroacetonitrile	357.7 ± 2.2	-
$CH_3CH_2CH_2SH$	1-propanethiol	354.2 ± 2.2	-
$CH_3CHSHCH_3$	2-propanethiol	353.4 ± 2.2	-
p-$CF_3C_6H_4NH_2$	4-(trifluoromethyl) aniline	353.3 ± 2.1	-
p-$CH_3C_6H_4OH$	p-cresol	350.3 ± 2.1	+
m-$CH_3C_6H_4OH$	m-cresol	349.6 ± 2.1	+
CH_3COOH	acetic acid	348.1 ± 2.2	+
HCOOH	formic acid	345.3 ± 2.2	+
$CH_3COCH_2COCH_3$	2,4-pentanedione	343.8 ± 2.1	+
$NCCH_2COOCH_3$	methyl cyanoacetate	340.8 ± 0.6	+
Reault		**351.8 ± 3.6**	

[a] Acidities are in kcal mol^{-1} and come from ref 38.

Chapter 4

Discussion

4.1 Conclusion

In aqueous solution and in the solid state, the canonical C1 tautomer predominates.[12, 13] Also, the C1 tautomer is the biologically relevant one, because in nucleosides, the deoxyribose moiety is attached to the N1 site of C1 cytosine.

However, in the gas phase, calculations and some experiments show that cytosine is found to coexist with some of its tautomers (Figure 1).[14-17] The order of the relative energies of these four most stable tautomers is consistent with previous calculations using density functional theory.[35] In this Discussion section, we will first discuss the experimental results of acidities and proton affinities in the gas phase. We will then discuss the presence of cytosine tautomers in the gas phase under our experimental conditions. Next, we will move on to discuss the deuterated experiments. At last, we will discuss the biological relevance of our experimental and computational results.

The summary of acidity and proton affinity studies of the most acidic and basic sites of cytosine is in Table 10. The average acidity of most acidic site in cytosine is 341.9 ± 2.9 kcal mol^{-1}. The average proton affinity of most basic site of cytosine is 227.9 ± 3.6 kcal mol^{-1}. The acidity of the less acidic site in cytosine is 351.8 ± 3.6 kcal mol^{-1} (Table 10). All these experimental results are consistent with the calculational results.

Table 10. Summary of acidity and proton affinity of most sites in cytosine

method	most acidic site (kcal mol^{-1})	most basic site (kcal mol^{-1})
Bracketing method	343.1 ± 2.9	228.7 ± 4.1
Equilibrium method 1	340.9 ± 2.1	228.1 ± 3.0
Equilibrium method 2	341.6 ± 2.3	227.7 ± 2.5
Cooks kinetic method	341.8 ± 2.1	227.1 ± 2.2
Experimental average	**341.9 ± 2.9**	**227.9 ± 3.6**

4.2 Tautomers

The more acidic site of cytosine is calculated to be 341-343 kcal mol^{-1} for C1, C2, C3 and 335 kcal mol^{-1} for C4. Therefore, a reference acid with acidity between 341 kcal mol^{-1} and 335 kcal mol^{-1} could be chosen to test the presence of C4. This reference acid will protonate C1, C2, C3 anions since it is more acidic than C1, C2 and C3 tautomers, but will not protonate all the C4 anions since it is less acidic than C4 tautomer. Also, the acidity of the reference acid is close to 335 kcal mol^{-1} to ensure the relatively faster completion of reaction between the reference acid and C1, C2 and C3 tautomers. The reference acid malononitrile (335.8 kcal mol^{-1}) is selected to react with the cytosine ions in the equilibrium method. The neutral malononitrile should protonate C1, C2, C3 anions with acidity at 341-343 kcal mol^{-1} but should not protonate C4 anions with acidity at 335 kcal mol^{-1}. We would expect some extent of cytosine anions left without reacting if the C4 tautomer is present. However, there are no cytosine anions present at long reaction times up to 60 s, which implies that the C4 tautomer is not present under our condition.

The more basic site of cytosine is calculated to be 227-229 kcal mol^{-1} for C1, C3, C4 and 219 kcal mol^{-1} for the C2 tautomer. A similar experiment as for acidity was carried out using the reference base *N*-methylaniline which has a proton affinity of 219.1 kcal mol^{-1}. Cytosine cations

are generated in the presence of neutral cytosine and *N*-methylaniline. We would expect a certain amount of cytosine cations to be left in tact (ie. Not undergo reaction) if the C2 tautomer is present. However, no cytosine cations are observed at long reaction times (60 s) implying the absence of the C2 tautomer. Since the C2 and C3 tautomers are close in energy and the barrier between them is quite low (less than 5 kcal mol^{-1}), both C2 and C3 can be excluded.[17]

Our results are not consistent with previous gas phase studies: C1 and C2 (and possibly C3) were found to coexist in a resonance enhanced multiphoton ionization (REMPI) experiment;[14] C1, C2 (and possibly C3) and C4 have been observed in IR matrix isolation studies;[15] C1, C2 and C4 tautomers were found to coexist in microwave (MW) spectroscopy studies.[16] One possible reason to explain why we did not observe the tautomers of cytosine is that other studies used higher energy methods to vaporize the solid cytosine. We usually heat up the solid probe to the lowest temperature that will vaporize solid cytosine (463-475 K). However, other methods might require the use of higher energy (the IR matrix isolation experiment used 500 K, and the MW spectroscopy study used 568 K).[15, 16] Our condition may only overcome the heat of sublimation of cytosine from the solid phase to the gas phase, but in other studies they might have extra energy to facilitate the tautomerism of the cytosine.

Therefore, we can conclude that only the C1 tautomer, which is the "canonical" structure in DNA, is predominant in the gas phase under our conditions. The acidities and proton affinities we measured above are the sites on the C1 tautomer. The experimental values of the most acidic site and most basic site are consistent with the calculational values of the C1 tautomer. The bracketing acidity of the less acidic site is close to the third acidic site value in the calculational results of C1 tautomer. A possible reason that we cannot bracket the second acidic site in cytosine is that the

acidity of the second acidic site is close to that of the most acidic site (only 3.3 kcal mol^{-1} difference by calculation).

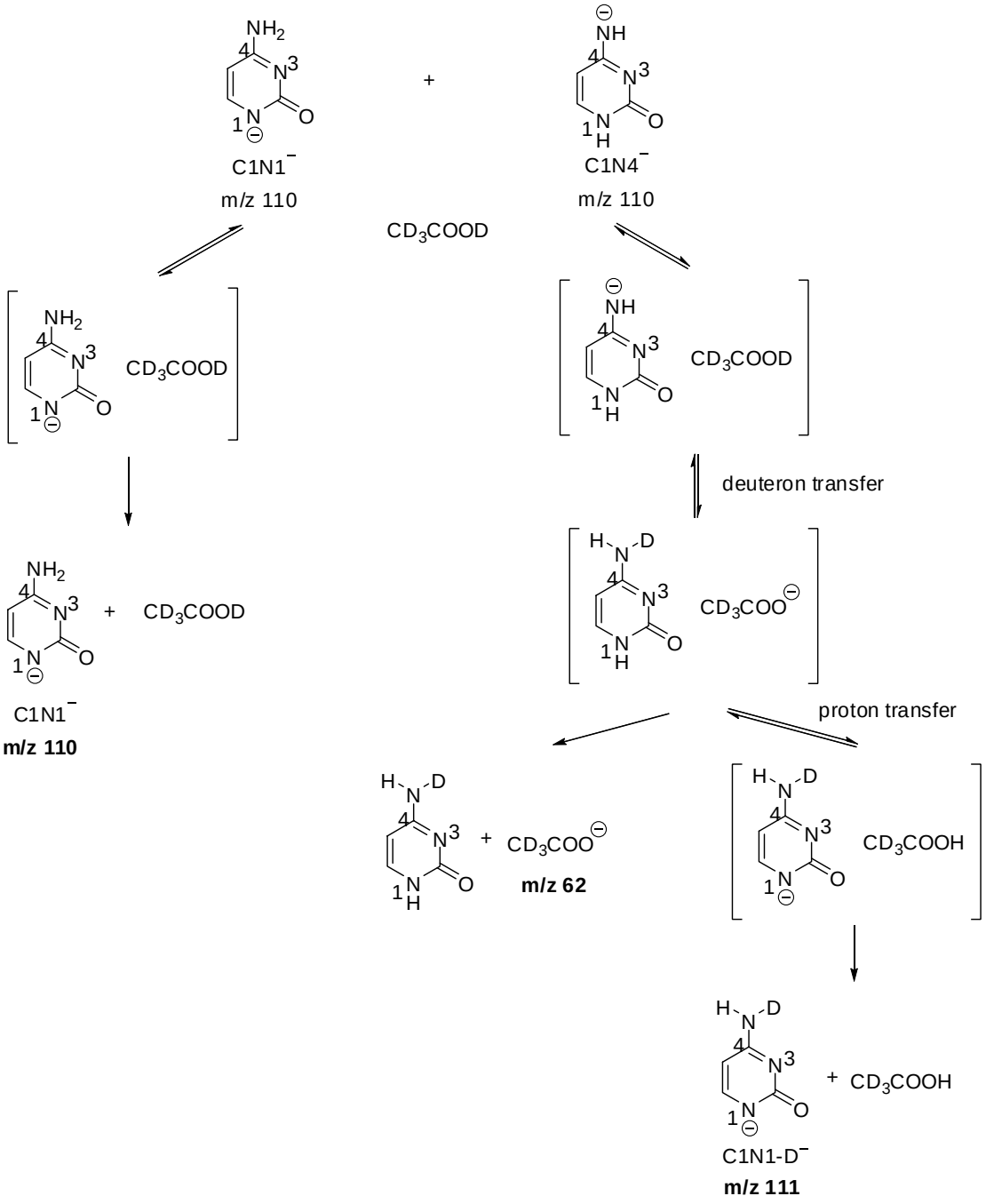
4.3 Deuterated Experiments

The purpose of deuterated experiments is to test the existence of the less acidic site of cytosine. We use two reference acids: deuterated formic acid (346 kcal mol^{-1}) and deuterated acetic acid (348 kcal mol^{-1}), whose acidities are stronger than the less acidic site, but weaker than the most acidic site.

Details of the design of the experiment are shown in Scheme 3, using deuterated acetic acid as an example. Under the less acidic site bracketing condition (ionization delay is 0.001s), both the C1N1 anion (deprotonated at the most acidic site) and the C1N4 anion (deprotonated at the less acidic site) are present in the cell. The C1N1 anion has the acidity around 342 kcal mol^{-1}, while the C1N4 anion has an acidity around 352 kcal mol^{-1} (Experimental Results). As deuterated acetic acid has an acidity of 348 kcal mol^{-1}, it could react with the C1N4 anion, but not with the C1N1 anion. The reaction between deuterated acetic acid and the C1N4 anion will produce deuterated cytosine anion (m/z 111). Therefore the presence of m/z 111 indicates the presence of the more basic $C1N4^-$ ion.

Under the less acidic site bracketing condition (ionization delay is 0.001s), the growth of peak with m/z 111 was observed for both DCOOD and CD_3COOD, indicating the presence of the less acidic site of cytosine. However, this peak was also observed with a longer ionization delay (up to 60 s). There should not be any less acidic site ions present under long ionization delay, so we should not see any peak 111 then. As the acidities of C1N1 and C1N4 are 342 kcal mol^{-1} and 352 kcal mol^{-1} respectively, we suspect that the two reference acids (DCOOD and CD_3COOD) we used

are too close in acidity to the most acidic site of C1, so they may also react with the most acidic site to generate the peak 111. Other deuterated reference acids with acidity between 348 kcal mol^{-1} and 352 kcal mol^{-1} could be used in the future to further study this problem.



Scheme 3

4.4 Biological Implications.

As noted before, TDG could excise mismatched nucleobases paired with guanine excluding cytosine.[18] However, the mechanism of this excision is unknown. Recently, Drohat and coworkers studied the relationship between the solution phase acidity of a series of nucleobase substrates and the kinetics of these substrates cleaved by TDG in solution.[18] Their studies indicate that the nucleobase is cleaved in its deprotonated form (the more acidic the nucleobase is, the easier the excision is).[18] Since the gas phase mimics the nonpolar environment of the active site in TDG, here we compare the gas phase acidity and proton affinity of cytosine, thymine and hypoxanthine to study why TDG might favor the cleavage of thymine and hypoxanthine over cytosine and which pathway the excision follows (Scheme 1).

4.4.1 Deprotonated nucleobase as a leaving group. Does the damaged nucleobase leave without prior protonation? The more acidic the damaged nucleobase is, the more easily the nucleobase anion leaves (Scheme 1A). The acidities of N1-H of cytosine, N1-H of thymine and N9-H of hypoxanthine were calculated at the B3LYP/6-31+G* level.[30, 39] Cytosine (ΔH_{acid} = 343.3 kcal mol^{-1}) is 11 kcal mol^{-1} less acidic than thymine (ΔH_{acid} = 332.3 kcal mol^{-1}) and 12.8 kcal mol^{-1} less acidic than hypoxanthine (ΔH_{acid} = 330.5 kcal mol^{-1}), which indicates that in the gas phase, thymine and hypoxanthine are much better leaving groups than cytosine if the damaged base leaves as an anion (pathway A).

4.4.2 Neutral nucleobase as a leaving group. Does the damaged nucleobase leave as a neutral? The more basic the nucleobase is, the more easily it will be protonated (Scheme 1B). The proton

affinities of the most basic site of cytosine, thymine and hypoxanthine were calculated at the B3LYP/6-31+G* level. Cytosine (PA = 226.5 kcal mol^{-1}) is 15.5 kcal mol^{-1} more basic than thymine (PA = 201.0 kcal mol^{-1}) and 6.9 kcal mol^{-1} more basic than hypoxanthine (PA = 219.6 kcal mol^{-1}) in the gas phase, which indicates that cytosine is more likely to be protonated and favors pathway B than thymine and hypoxanthine.

However, the fact is that TDG cleaves thymine and hypoxanthine but avoids cytosine, so the mechanism of TDG probably takes pathway A in which the nucleobase leaves as an anion (gas phase acidity relevant) since thymine and hypoxanthine are better leaving group than cytosine.

4.5 Conclusions

Only the C1 tautomer is predominant in the gas phase under our conditions. The acidity and proton affinity of cytosine obtained from different methods (calculation, bracketing, equilibrium and Cooks method) are consistent with each other. The TDG enzyme cleaves damaged or mismatched nucleobases by pathway A in which the nucleobase leaves as an anion.

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