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Stability of DNA Duplexes Containing Hypoxanthine (Inosine): Gas versus Solution Phase and Biological Implications

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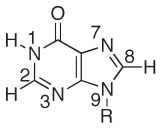
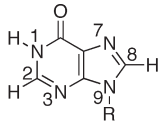


hypoxanthine (inosine)

A series of 9-mer DNA duplexes of the sequence 5′-d(GGTT**X**TTGG)-3′/3′-d(CCAA**Y**AACC)-5′, where the central base X or Y = adenine (A), guanine (G), thymine (T), cytosine (C), and hypoxanthine (H), have been examined toward understanding the effect of hypoxanthine on DNA stability. Comparison of the duplex stability in the gas phase versus solution indicates that hypoxanthine has much less of a destabilizing effect in the gas phase versus in solution, relative to the normal complementary duplexes. The biological implications of these results, both in the context of hypoxanthine as a universal base and as a damaged base, are discussed.

Introduction

Hypoxanthine (**1a**) is a nucleobase that occurs naturally in tRNA and is a key intermediate in the de novo biosynthesis of purine nucleotides; it is also a mutation that occurs in DNA when adenine is deaminated.[1,2]



1 **a** R = H (hypoxanthine)
b R = (deoxy)ribosemonophosphate (inosine)

Hypoxanthine (also called "inosine" in its nucleotide form (**1b**)) is often referred to as a "universal base". This ability was first recognized after the discovery that inosine was often present in the first anticodon position in various tRNA sequences.[2] That first anticodon position pairs to the third codon position on mRNA, which is proposed to have some "play" that allows for non-Watson−Crick base pairs (such as hypoxanthine·adenine); this is the well-known Crick "wobble hypothesis".[2] As a potentially useful universal base, hypoxanthine can form base-pair structures with all the normal nucleobases (Figure 1).[3,4]

Universal bases have a myriad of potential uses in molecular biology.[3,5,6] In many applications, a needed oligonucleotide sequence target may not be exactly known and the universal base can act as a "wild card" that will bind to any nucleobase indiscriminately.[5,7−9] For example, hypoxanthine residues have been placed at ambiguous points in oligonucleotide probes that screen genomic DNA libraries.[9−13] Universal bases are of particular utility when probes and

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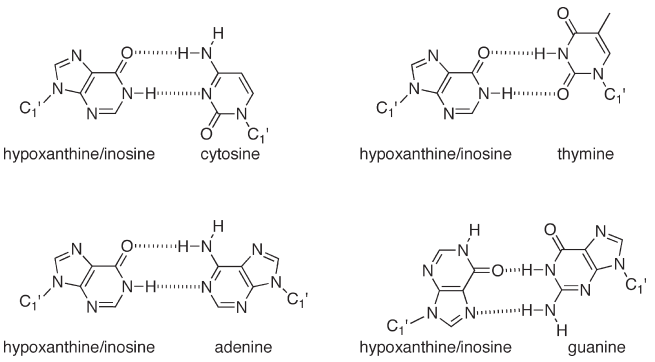

FIGURE 1. Possible structures of hypoxanthine·normal base pairs.

primers are designed based on the amino acid sequence of a protein, which can be complicated by codon degeneracy or fragmentary peptide sequence data.[5,7,9−11,14,15]

Although hypoxanthine has been used in primers and probes, the fundamental examination of the stability of base pairs containing it has been limited; the major experimental study was conducted by Watkins and SantaLucia in 2005.[3,6,7,13] They examined the solution phase stability (melting temperature) of 84 dimers containing hypoxanthine; combining their data with that of 13 additional oligonucleotide dimers that were examined previously in the literature, they were able to characterize the hypoxanthine nearest-neighbor parameters, which allows for accurate prediction of the stability of oligonucleotides containing hypoxanthine. Overall, in solution, melting temperatures of duplexes containing hypoxanthine vary widely and are on average lower than those for complementary duplexes containing only adenine, guanine, cytosine and thymine.[3,6,7,13] Also, hypoxanthine·cytosine is more stable as a base pair than other combinations; hypoxanthine is therefore not so universal that it binds equally to each natural nucleobase in solution.[3,5,7]

In the gas phase, numerous computational studies and limited experimental studies have explored the tautomeric, acidic and basic properties of hypoxanthine, as well as the various hydrogen-bonded dimers.[16−28] As far as we know, however, no gas-phase experimental study of the effect of hypoxanthine on duplex stability has been carried out.

Our interest is in examining the gas-phase properties of hypoxanthine. In our work, we have found that gas phase behavior often differs from that in aqueous solution and that reactivity in the absence of solvent can be useful as a starting point for extrapolation to other media. For example, we have shown that the leaving group ability of certain damaged bases is enhanced in a nonpolar environment, relative to in aqueous solution.[28−37] We hypothesize that Nature could take advantage of this enhancement by providing a nonpolar enzyme environment to help discriminate damaged from normal bases for cleavage. Because the interior of ribosomes, polymerases, the cell plasma, and other biological media in which universal bases may have a role vary in polarity and are not fully aqueous, gas-phase studies of hypoxanthine could lend insight into its biological role.

In this work, we examine the effect of hypoxanthine on the gas-phase stability of a series of DNA duplexes using mass spectrometry (MS) and compare those values to solution to evaluate the effect of hypoxanthine on duplex stability in nonaqueous environments. We discuss our results in the context of hypoxanthine as a possible universal base and also in its role as a damaged, mutagenic base.

Results and Discussion

We examined the 9-mer 5′-d(GGTT**X**TTGG)-3′/3′-d(CCAA**Y**AACC)-5′, where the central base X or Y = adenine (A), guanine (G), thymine (T), cytosine (C), and hypoxanthine (H). We chose this particular sequence because we have extensively characterized this series (where X or Y = all possible combination of A, G, T, and C) in both the gas and solution phases in earlier studies from our laboratory.[34,38−40] We have found that this 9-mer is long enough to form helical structures but also manifests measurable changes in stability when just the central base pair is changed.[34,38−40] Furthermore, these duplexes, because of the terminal GC base pairs that help maintain helical structure during dissociation, are particularly well-suited to the traditional two-state dissociation model that allows for accurate theoretical prediction of melting temperatures.[41] In addition to our prior work in studying DNA duplex stability in the gas phase, there are studies from several other laboratories as well that serve to validate the methods used herein.[34,38,42−46]

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TABLE 1. Experimental ($T_{m,expt}$) and Calculated ($T_{m,calc}$) Solution-Phase and Gas-Phase (E_{50}) Data for XY Duplexes

XY	$T_{m,expt}$ (°C)	$T_{m,calc}$ (°C)	E_{50} (%)
GC	35.83	33.48	11.75 ± 0.10
CG	34.00	31.98	11.40 ± 0.09
TA	31.78	29.32	10.82 ± 0.08
AT	29.49	26.54	10.84 ± 0.07
CH	29.33	26.97	10.99 ± 0.04
HC	28.43	26.40	11.39 ± 0.03
AH	23.78	20.60	10.77 ± 0.05
HT	19.78	18.87	11.06 ± 0.09
HA	17.33	15.88	10.82 ± 0.07
TH	14.89	8.68	11.03 ± 0.05
HG	14.67	8.66	11.37 ± 0.04
GH	13.89	13.21	11.21 ± 0.04
HH	13.67	10.31	11.01 ± 0.08

To simplify the nomenclature of each duplex in this paper, we use only the variable central base of each strand to represent the whole duplex. For example, a duplex called "GH" refers to the duplex 5′-d(GGTT**G**TTGG)-3′/3′-d(CCAA**H**AACC)-5′, where X = guanine (G), Y = hypoxanthine (H). The various combinations where X or Y is an H can form nine duplexes (AH, CH, GH, TH, HH, HA, HC, HG and HT); we studied all of these plus the four complementary duplexes GC, CG, AT and TA.

Solution-Phase Stability. We assess the solution-phase stability using two methods: the traditional method for measuring the solution-phase stability of a DNA duplex, melting temperature (T_m), and using a gas-phase method that yields what we refer to as duplex stability (DS%). For T_m, when the temperature of a DNA duplex solution is slowly increased, the ordered double helical structures dissociate into single strands. The midpoint of this transition is the T_m. The higher the T_m, the more stable the duplex. The experimental ($T_{m,expt}$) and calculated ($T_{m,calc}$) solution melting temperatures for our XY duplexes are shown in Table 1 (second and third columns, ordered in decreasing $T_{m,expt}$).[3,7,47] Methods have been developed that quite accurately predict solution-phase melting temperature.[3,7,47,48] Our experimental and calculated results correlate linearly with an R^2 of 0.97. The normal duplexes (GC, CG, TA, and AT) are significantly more stable than those containing hypoxanthine, with the exception of CH and HC, which are close in stability to AT. As expected, the duplexes containing G and C are more stable than those containing A and T (the former has three hydrogen bonds while the latter has only two). Among the hypoxanthine duplexes, CH is the most stable and HH is the least stable.

In previous work, we showed that when X and Y are normal nucleobases, the duplex ion abundance resulting from electrospray (which we term DS%) reflects the solution-phase stability.[34,38] That is, if the electrospray process should volatilize the duplexes with relative integrity, then the resultant mass spectrum should be a "snapshot" of the solution-phase composition and the relative ion abundances among a series of duplexes with differing stability should reflect their relative solution-phase stabilities.[49−56] In order to assess whether this method of using ion abundances to measure relative solution-phase stabilities can be generalizable to DNA duplexes containing a damaged base such as hypoxanthine, we plotted the duplex ion abundances versus the solution phase T_m values (Figure 2). A reasonable linear relationship is seen (R^2 = 0.93); thus, for this series of duplexes, monitoring the duplex ion signal is a fast and efficient method for assessing relative solution phase stabilities. This is the first study establishing a correlation between solution-phase stability and mass spectrometric signal abundance for mismatched duplexes containing a damaged base. Using MS in this way does have limitations, since ion intensity is dependent on many factors, but we show that for a controlled series such as this the method does work, even if the nucleobases involved are abnormal.

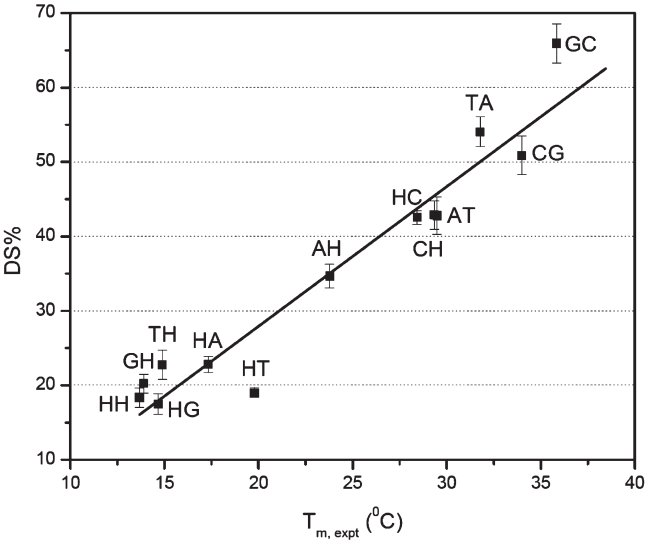

FIGURE 2. Plot of DS% versus $T_{m,expt}$ for complementary duplexes and duplexes containing hypoxanthine (5′-d(GGTT**X**TTGG)-3′/3′-d(CCAA**Y**AACC)-5′).

Gas-Phase Stability. Whereas ion abundance yields the solution-phase stability, collision-induced dissociation (CID) to dissociate a duplex into its single strand components yields gas-phase stability.[34,38,42−46,57] Electrospray of these 9-mer sequences yields duplex ions with both −3 and −4 charges. Under gentle CID conditions, we find that

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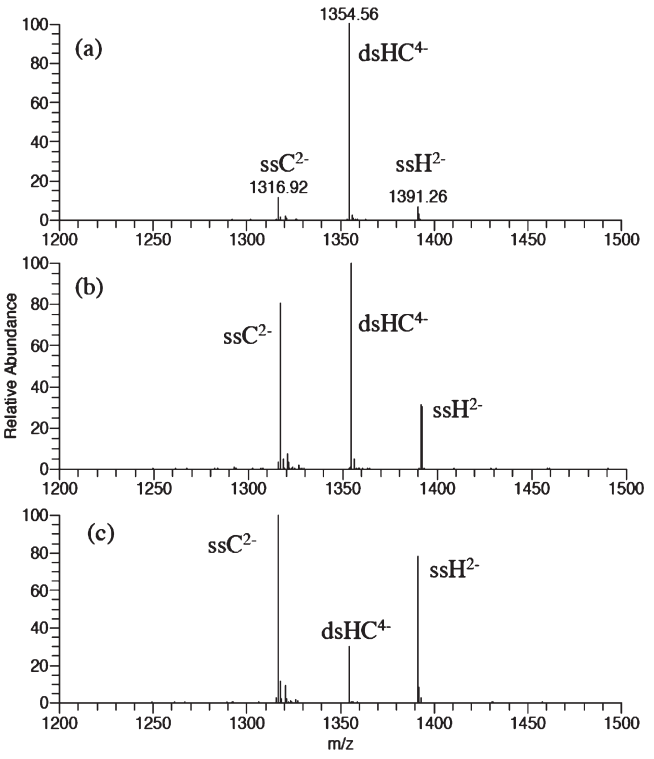

FIGURE 3. CID spectra of the duplex HC^{4-} ions at relative collision energies of (a) 10.4%, (b) 11.2%, and (c) 12.0%: "ds" indicates double strand; "ss" indicates single strand.

dissociation of the −4 duplex into its constitutive single strands (−2 charge on each) is the major fragmentation pathway (Figure 3). The dissociation of the −3 charged duplexes preferentially yields extensive cleavage of covalent bonds, with little noncovalent dissociation. The effect of charge state on duplex stability has been discussed previously by others, and stability comparison among different ions is valid only if all the duplexes have the same charge states and fragmenting routes.[42,57−62] Therefore, we report the CID experiments on the −4 charged duplexes only, where duplex dissociation into the two single strands is the major pathway.

The dissociation of the parent duplex ion is monitored by the disappearance of the duplex signal and the appearance of the single strands. The collision energy at which 50% of the duplexes are dissociated into single strands ("E_{50}"; more details in Experimental Section) is used to characterize the gas-phase stability.[34,38,42−46,57] The dissociation profiles of four duplexes (GC, AT, HC, and HT) are displayed in Figure 4. To achieve the same degree of dissociation among all these duplexes (as indicated by the E_{50} value), the GC duplex (squares) requires the highest collision energy (as indicated by the largest E_{50} value), followed by HC then HT (circles and upright triangles), with AT (upside down triangles)

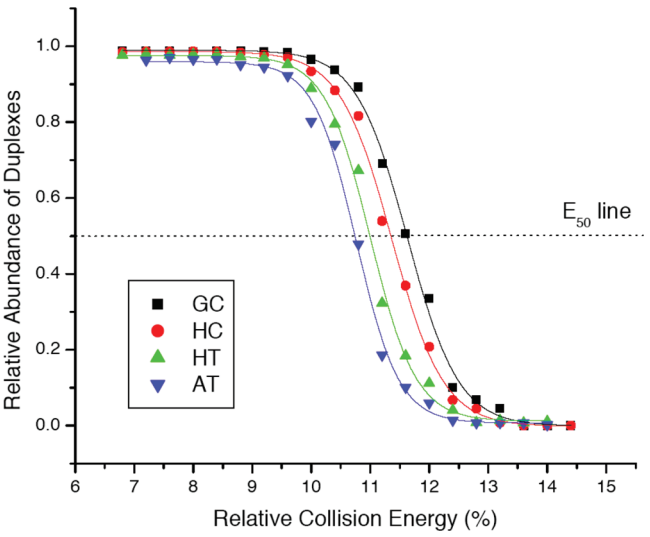

FIGURE 4. Gas-phase dissociation profiles of four XY duplexes: GC, HC, HT, and AT.

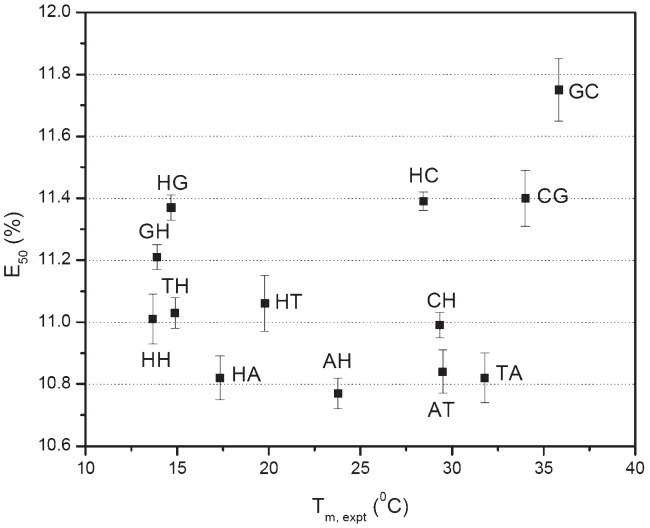

FIGURE 5. Comparison of gas-phase stability (E_{50}) and solution-phase stability ($T_{m,expt}$) of the 13 XY duplexes. Error bars indicate the standard deviation for each E_{50} value.

having the lowest E_{50} and therefore being the least stable. This would indicate that the gas-phase stability order of these four duplexes is GC > HC > HT > AT.

Table 1 (fourth column) lists all the E_{50} values for the 9 XY duplexes containing H and the four complementary duplexes, together with the melting temperatures, to allow for direct comparison between gas-phase and solution-phase stabilities. These data are also plotted in Figure 5. The E_{50} difference between the most stable GC duplex and the least stable AH duplex is only 0.98%. However, these differences are significant because the average standard deviation is only 0.06%. From Figure 5, one can see that the solution- and gas-phase stabilities do not track (R^2 value is 0.07). Many of the hypoxanthine-containing duplexes (HC, HG, GH, HT, TH, HH, CH) are more stable than the normal duplexes AT and TA.

We believe that this disparity between the solution- and gas-phase stabilities is meaningful. In previous work, we and

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TABLE 2. $T_{m,expt}$ **and** E_{50} **Data for XY Duplexes (5′-d(GGTTXTTGG)-3′/3′-d(CCAAYAACC)-5′)**

XY	$T_{m,expt}$ (°C)		E_{50} (%)	
GC or CG	35.83 (GC)	34.00 (CG)	11.75 ± 0.10 (GC)	11.40 ± 0.09 (CG)
AT or TA	29.49 (AT)	31.78 (TA)	10.84 ± 0.07 (AT)	10.82 ± 0.08 (TA)
HC or CH	28.43 (HC)	29.33 (CH)	11.39 ± 0.03 (HC)	10.99 ± 0.04 (CH)
HG or GH	14.67 (HG)	13.89 (GH)	11.37 ± 0.04 (HG)	11.21 ± 0.04 (GH)
HT or TH	19.78 (HT)	14.89 (TH)	11.06 ± 0.09 (HT)	11.03 ± 0.05 (TH)
HA or AH	17.33 (HA)	23.78 (AH)	10.82 ± 0.07 (HA)	10.77 ± 0.05 (AH)
HH	13.67 (HH)		11.01 ± 0.08 (HH)	

others have shown that gas- and solution-phase stabilities often *do* track,[34,38,42−46] yet here they do not. Although the two do not track, there are some clear trends (Table 2). In Table 2, we arrange the data so that the duplexes that do and do not contain H can be more easily compared. In solution, all duplexes containing hypoxanthine are less stable (have lower melting temperatures) than the normal GC ($T_{m,expt}$ = 35.83 °C), CG (34.00 °C), TA (31.78 °C), and AT (29.49 °C) duplexes. This is consistent with earlier studies pointing to a wide range in stability in hypoxanthine duplexes, with hypoxanthine clearly favoring cytosine for base pairing.[3,6,7,13] In the gas phase, however, the hypoxanthine-containing duplexes are *not*, generally, less stable than the normal duplexes. In fact, the E_{50} values for those duplexes containing hypoxanthine fall, for the most part, between the E_{50} values of TA (E_{50} = 10.82%) and GC (11.75%). (AH is the only duplex that may be less stable than TA, with an E_{50} of 10.77 ± 0.05). Therefore, in the nonpolar environment of the gas phase, the hypoxanthine-containing duplexes are not unusually unstable but rather fall into a range between the complementary GC and TA duplexes.

This disparity between the effect of hypoxanthine in solution versus in the gas phase is likely due to differing base stacking rules in the two media. In previous work, we showed that if a series of duplexes had the same number of hydrogen bonds (same GC content), the solution- and gas-phase stabilities do not always correlate.[34] We hypothesized that when the GC percentage is constant in a series of duplexes, hydrogen-bonding effects should be similar and any differences in stability between the solution and the gas phase are thus attributable to stacking.[34]

Hypoxanthine has been shown to be an effective base stacker; gas-phase calculations indicate that stacking interactions with hypoxanthine are as large as the best stacking interactions between normal bases.[16] We hypothesize that the disparity between the solution and gas-phase results is due, at least in part, to changes in base stacking ability of hypoxanthine with the various other nucleobases in the different media.

Biological Implications. Hypoxanthine as a Universal Base. The most striking aspect of our data is the disparity between the solution- and gas-phase stabilities. Our data indicate that in the nonpolar environment of the gas phase, the hypoxanthine-containing duplexes are not unusually unstable but rather fall into a range between the complementary GC and TA duplexes (Table 2, third column, E_{50} data). This difference in hypoxanthine's effect on duplex stability (relative to normal complementary duplexes) in a nonpolar versus a polar environment could be useful in applications utilizing hypoxanthine as a universal base. The differences we see could explain why although hypoxanthine (inosine) is a key component in the anticodon−codon wobble pairing of tRNA and mRNA (which occurs in the ribosome), it has had mixed success as a universal base in in vitro applications such as PCR primers and hybridization probes (where its wide-ranging effects on duplex stability make it not so universal).[2,3,5,7] In applications that might occur in media that are not aqueous solution (for example, in inhibitory antisense strands designed to bind to mRNA in vivo), hypoxanthine may be a better universal base.[1,15] Of course, our fundamental studies are at the extreme (the gas phase), but these results point to interesting differences in the intrinsic stability of duplexes versus those that are in solution.[5,15]

Hypoxanthine as a Damaged Base. Hypoxanthine can result from the deamination of adenine and as such is a damaged base in DNA and a mutagenic agent.[1,2] The mutagenicity of hypoxanthine lies in its ability to cause an A·T to G·C transition.[63,64] If the adenine in an A·T base pair is deaminated, the base pair becomes an H·T base pair. In replication, H prefers C, so once the duplex unwinds and replicates, an H·C base pair is formed. When that duplex replicates, the C will base pair with a G; this is the A·T to G·C transition. Since the specific sequence of the human genome is responsible for coding proteins, signaling, and a myriad of other important functions, the hypoxanthine mutation can be deleterious.

The human genome is protected by an enzyme, alkyladenine DNA glycosylase (AAG), which excises hypoxanthine from DNA.[65−67] Previous studies from our laboratory on "free" nucleobases (i.e., the purine base alone, not incorporated into DNA) indicate that AAG may provide a nonpolar environment that aids in the discrimination of damaged from normal nucleobases.[28−37] We wanted assess whether these current stability studies are consistent with this hypothesis.

AAG will excise hypoxanthine from all possible base pairs (hypoxanthine hydrogen bonded to adenine, guanine, thymine, and cytosine). It appears that hypoxanthine is more efficiently excised from H·T base pairs than H·C base pairs, which is often explained by the solution-phase observation that H·T base pairs are less stable than H·C base pairs (so it would be easier to excise a hypoxanthine paired with a thymine). However, this theory is inconsistent with the observation that hypoxanthine is more efficiently excised from H·C base pairs than H·G and H·A base pairs even though H·C base pairs are *more* stable than H·G and H·A base pairs in solution.[68] These excision preferences are further complicated by the limited set of sequences studied with AAG, and the probability that the preferences are also controlled by the nucleobases flanking each base pair (the nearest neighbors).[68−71]

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Our solution-phase results are consistent with others' solution-phase observations to date in that for our sequence 5′-d(GGTT**X**TTGG)-3′/3′-d(CCAA**Y**AACC)-5′, HC, and CH are more stable than HT and TH (Table 2, second column, $T_{m,expt}$ data). Also, HC and CH are more stable than the same sequences containing G and A. Therefore, our solution-phase results do not explain observed AAG excision preferences either.

The question is, do our gas-phase stabilities better explain the observed AAG excision preferences? We find that there is some consistency, though the sequence appears to have a strong effect. For example, the HT duplex is less stable than the HC duplex, which is consistent with the preferred excision of H from H·T over H·C base pairs (Table 2, third column, E_{50} data). But the CH and TH duplexes are actually quite comparable in stability. That is, although both HC and CH have the same sequence, and HT and TH have the same sequence, the relative stabilities among them are different. This is due to the differences in base stacking ability of H when it is in the "X" versus the "Y" position in the 5′-d(GGTT**X**TTGG)-3′/3′-d(CCAA**Y**AACC)-5′ sequence (i.e., flanked by thymines versus adenines). In another example, the CH duplex is less stable than the GH duplex, which is also consistent with the more efficient excision of H from H·C over H·G base pairs. However, the HC and HG duplexes are similar in stability. Therefore, the gas-phase data are not entirely consistent with known AAG reactivity, but clearly, the preferences are sequence dependent. Also, the gas-phase data are quite different from the solution-phase data. These differences are tantalizing in that it may be possible that an explanation for AAG preferences might be better understood by comparing and contrasting solution-phase and intrinsic gas-phase stabilities. However, because AAG excision preferences are sequence-dependent,[68−71] and our sequence is different from those studied with AAG (which is also a limited set), we cannot draw any definitive conclusions yet. We intend to conduct future work with sequences that have been studied with AAG to ascertain whether the observed AAG preferences might correlate to the gas-phase stabilities.

Last, it has been found that excision of hypoxanthine is much lower when hypoxanthine is flanked by a 5′G and a 3′C versus a 5′T and a 3′A.[69] In our studies, hypoxanthine was either flanked by 5′ and 3′ adenines, or 5′ and 3′ thymines. In the future, we would like to ascertain how gas-phase stabilities of duplexes containing hypoxanthine with different nearest neighbors compare to solution-phase stabilities. Such data will be helpful both in the context of AAG and also for further assessing the utility of hypoxanthine as a universal base.

Conclusions

Comparison of the gas-phase and solution-phase stabilities of the 13 XY duplexes (5′-d(GGTT**X**TTGG)-3′/3′-d(CCAA**Y**AACC)-5′) where X, Y = A, G, T, C, and H indicates that although hypoxanthine has a fairly consistent destabilizing effect in aqueous solution, in the gas phase, those DNA duplexes with hypoxanthine are for the most part as stable or more stable than the normal AT and TA duplexes (and less stable than the GC and CG duplexes). The comparable stability of the hypoxanthine-containing duplexes relative to the normal duplexes in vacuo could mean that hypoxanthine, which has limitations as a universal base in vitro, might prove useful in in vivo applications where the environment may not be aqueous.[5] Our results also could potentially lend insight into hypoxanthine's role as a damaged base; when hypoxanthine arises from deamination of adenine, it is excised by the enzyme AAG. The basis for the excision preferences of AAG for certain base pairs is not understood. Our current gas-phase data provide tantalizing results that differ from the solution-phase data and imply possible insight into AAG excision preferences. Future studies will probe whether excision efficiencies of hypoxanthine from specific sequences correlate to gas-phase stabilities of the hypoxanthine-containing base pairs. Nearest neighbor effects on the stability of duplexes containing hypoxanthine in the gas phase versus in solution will also be studied.

Experimental Section

Sample Preparation. Oligodeoxynucleotide single strands were purchased from a commercial supplier. These single strands were predesalted and used without further purification. Stock solutions of 62.5 μM duplex were prepared in 40 mM NH_4OAc aqueous solution at pH 7.0. The stock solutions were then annealed at 90 °C for 10 min and cooled slowly to 0 °C. Before injection into the ESI-MS, the stock solutions were diluted to 12.5 μM in 40 mM NH_4OAc mixed with 20% methanol.

Melting Temperature in Solution. The $T_{m,expt}$ values were measured from UV melting curves obtained using a spectrophotometer. All the measurements were performed at a duplex concentration of 12.5 μM in 40 mM NH_4OAc. Melts were monitored at 260 nm by increasing the temperature continuously from 0 to 80 °C, with absorbance measured every 0.2 °C. The estimated precision for the melting temperature experiments is 0.3 °C.[40]

The $T_{m,calc}$ values of the DNA duplexes are predicted by the program "MELTING".[47] The settings are as follows: (1) hybridization type: dnadna (for a DNA duplex); (2) nearest neighbor parameters set: all97a.nn; (3) salt concentration: 0.04 M; (4) nucleic acid concentration (total): 25.0 μM; (5) nucleic acid correction factor: 4 (for non-self-complementary duplex); (6) salt correction: san98a; (7) nearest neighbor parameters for inosine mismatches: san05a. The error for estimating T_m values by the MELTING program is ± 1.6 °C.[3,7,41,47,72,73]

ESI-Quadrupole Ion Trap Mass Spectrometer and "E_{50}" Experiments. Negative-ion ESI-MS spectra were obtained with a quadrupole ion trap. The 0 °C solution was infused at 25 μL/min directly into the mass spectrometer. The spray voltage was −4.0 kV, while the capillary temperature was 175 °C. Collision-induced dissociation (CID) was performed in the mass analyzer by varying the relative collision energy with a default activation time of 30 ms and a q value of 0.25. The applied collision energy is a normalized collision energy (in %) that corrects for the m/z dependence of the activation voltage required for ions of different m/z ratios.[58] The range of amplitude applied in the MS/MS studies is 0.4−1.0 V.[58] The gas-phase stability of the duplexes is measured in a relative way by subjecting the duplex

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parent ions to increasing collision energies during the CID event in an ion trap. E_{50} is defined as the collision energy at which 50% of the duplexes are dissociated into single strands, and is used to characterize the gas phase stability.[34,38,43−46] A higher E_{50} corresponds to a more stable duplex in the gas phase. Although CID is a kinetic experiment, the dissociation is assumed to be endothermic enough such that the barrier and the endothermicity are similar.[34,38,44,52,74−76] The term coined by Gabelica and DePauw for this sort of measurement is "kinetic stability".[44] When we refer to gas-phase stability in this paper, we therefore mean kinetic stability. The internal energy distribution of the parent ion is poorly defined due to the multiple collision events in the ion trap. We therefore do not intend to report absolute duplex dissociation energies but rather relative gas-phase stabilities as reflected by the E_{50}'s, a method established previously by our group and others.[34,38,42−46,57,58,77]

Experimental conditions were tuned by optimizing the −4 charged duplex ions of the TA duplex (5′-d(GGTTTTTGG)-3′/3′-(CCAAAAACC)-5′) (m/z = 1358); the conditions thus obtained were applied to all the duplexes. Duplex abundance is normalized by using the equation: % duplex (DS%) = (2 × [all duplexes]) /([all single strands] + 2 × [all duplexes]), where the values in brackets are absolute ion abundances.[34,38,43−46] The reported duplex abundance is an average of six full-scan measurements; the average standard deviation is 1.69%. Duplex dissociation profiles were fitted with sigmoid equations, and the corresponding E_{50} values were derived using Origin 6.0 software.[78] Each CID experiment was performed under a parent ion isolation width of w = 5; in previous studies we show that changing the isolation width does not change relative E_{50} values.[34,38] The reported E_{50} value for each XY duplex is an average of six measurements. The average standard deviations for the measurement of E_{50} for all the XY duplexes are 0.06%.

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