Rare Tautomer Hypothesis Supported by Theoretical Studies: Ab Initio Investigations of Prototropic Tautomerism in the *N*-Methyl-P Base

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Density functional theory calculations were applied to the prediction of the tautomeric properties of *N*-methyl-P (6-methyl-3,4-dihydro-8*H*-pyrimido[4,5-*c*][1,2]oxazin-7-one), a base of the nucleoside analogue dP (6-(2-deoxy-β-D-ribofuranosyl)-3,4-dihydro-8*H*-pyrimido[4,5-*c*][1,2]oxazin-7-one), for which water-solution experimental data have become available recently. The calculations have been performed for three tautomers in the gas phase, with various numbers of water molecules, and within the polarizable continuum model (PCM) of solvation. The obtained results correctly predict the presence of two tautomers and reproduce accurately the experimentally obtained ratio of the two most stable tautomeric forms when using a combination of explicit water molecules and the PCM of solvation. This lends additional support to the rare tautomer hypothesis of substitution mutagenesis in DNA replication.

Introduction

It is widely accepted that mutations in vivo may proceed though the formation of base mispairs during DNA replication. However, there are still a lot of mechanisms of DNA mutations proposed in the literature.[1] Among them historically, the first was the idea suggested by Watson and Crick[2] and later elaborated by Topal and Fresco.[3] According to Watson and Crick, the fidelity of DNA synthesis or, in other words, the probability of point spontaneous mutations depends on the possibility of DNA bases to form the so-called rare tautomeric forms. This kind of point spontaneous mutations occurs through the proton transfer in nucleic acid bases and the formation of rare tautomers. These tautomers are able to form base pairs with the bases that are not complementary to the canonic forms and, therefore, lead to mutations (Figure 1). This theory is known as the "rare tautomer hypothesis" of substitution mutagenesis. It should also be highlighted that to date there has been no direct evidence of mispairs involving tautomeric forms of DNA bases. In contrast, the evidence of cytosine deamination,[1,4] base oxidation,[1,5] and alkylation[1,6] as well as the formation of wobble and ionized or protonated DNA bases,[1,7] which are also considered the source of spontaneous mutations, is known. Therefore, some of the sources and nature of the DNA base mispairing are still being argued.

The main source of information about the properties of rare tautomers are the results of gas phase and inert gas matrix experiments[8] as well as quantum-chemical calculations.[9] These studies have been performed to estimate the relative stabilities of different tautomers of all nucleic acid bases. It was found that some bases have rare tautomeric forms that possess similar or even greater stability than their canonic forms. Thus, for example, it was found that cytosine primarily exists in the form of two tautomers—canonic and hydroxo forms—with a possible small presence of the imino tautomeric form.[8c−h,9a,b] Similar findings have been published for guanine[8o−q,9c−h] and all other nucleic acid bases. As already mentioned, all of the experimental studies have been performed in the gas phase, while there is some evidence that the bases are hydrated during the DNA functioning in living cells by at least a limited amount of water molecules.[10]

The experimental data on the tautomeric properties of DNA bases in water solution are not known. The estimations[3] suggest that the constant of tautomeric equilibrium should be significantly lower than the one corresponding to the gas phase, since the canonic forms are stabilized more by a polar surrounding. These data are also confirmed by recent quantum-chemical calculations.[11] Thus, the experimental determination of tautomerism of nucleic acid bases in a polar medium is of great importance.

The first support of the proposal that the minor tautomeric forms of the natural bases may play an important role in substitution mutagenesis during DNA replication appeared recently[12] in the study of the nucleoside analogue dP (6-(2-deoxy-β-D-ribofuranosyl)-3,4-dihydro-8*H*-pyrimido[4,5-*c*][1,2]-oxazin-7-one). In this study, the tautomeric constant (the equilibrium constant of tautomer interconversion) of *N*-methyl-P (6-methyl-3,4-dihydro-8*H*-pyrimido[4,5-*c*][1,2]oxazin-7-one)—a model compound of the nucleoside dP—has been evaluated spectrophotometrically in aqueous solution. Like natural nucleic acid bases, dP can exist in several tautomeric forms and form base pairs with different natural bases such as adenine (A) in its imino form or guanine (G) in its amino form (Figure 2). It was found that, in aqueous solution, *N*-methyl-P (6-methyl-3,4-dihydro-8*H*-pyrimido[4,5-*c*][1,2]oxazin-7-one) exists mostly in the form of an imino tautomer with a ratio of imino to amino tautomeric forms of 11:1, which is in contrast to the tautomeric properties of DNA bases in water where the rare-to-canonic ratio is estimated[3] to be in the range 10^{-5}−10^{-4}. In the study, the authors compared the tautomeric constant of *N*-methyl-P with the incorporation properties of the nucleotide dPTP. Interest-

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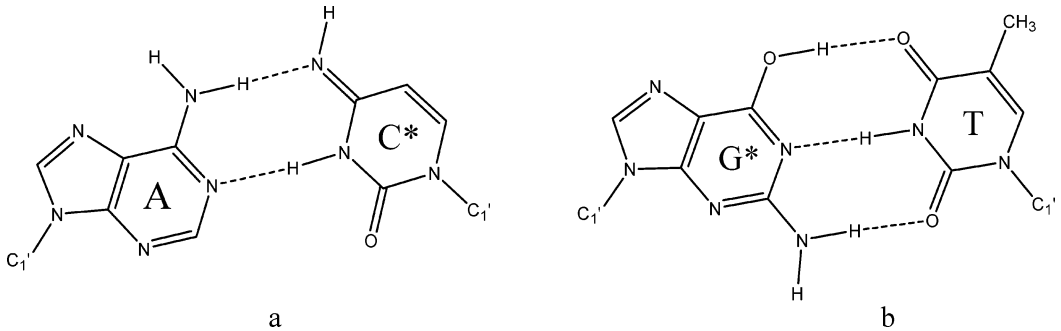

Figure 1. Examples of base mispairing in rare tautomeric forms: (a) adenine (A) pairs with imino tautomer of cytosine (C*); (b) thymine (T) pairs with enol tautomer of guanine (G*).

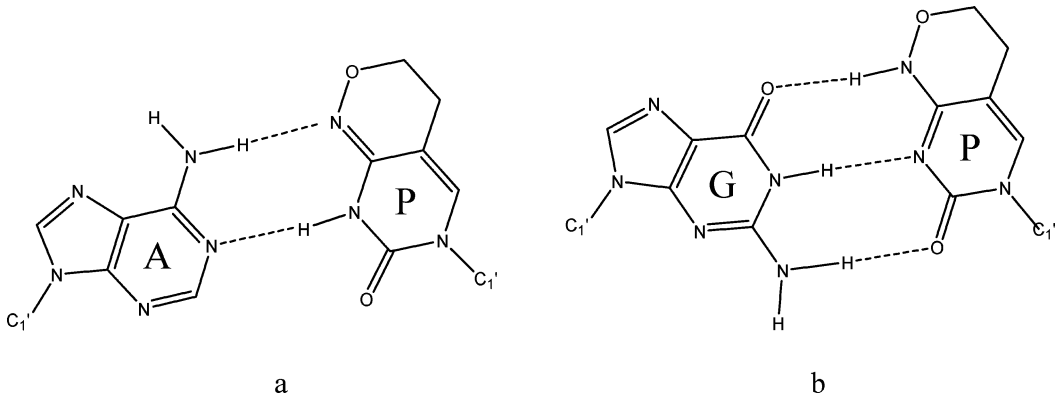

Figure 2. Examples of base pairing of P with (a) adenine (imino tautomer of P) and (b) guanine (amino tautomer of P).

ingly, the ratio of relative catalytic efficiencies for the incorporation of dPTP opposite A versus G was 10.6:1.0, which is almost the same as the concentration ratio of the two tautomeric forms of *N*-methyl-P. Thus, the results indicate that, in the absence of proof-reading, a direct correlation exists between the tautomeric constant and nucleotide incorporation preference by the Klenow polymerase. This lends experimental support to the rare tautomer hypothesis for substitution mutagenesis.

In this study, we have performed a theoretical study of the *N*-methyl-P tautomerization using high-level quantum-chemical methods and have compared them to the experimental results. A close agreement between the experimental findings and the results of the quantum-chemical calculations provides an independent support for the results obtained in the experimental study[12] and also gives credence to previous theoretical results of the tautomeric properties of nucleic acid bases in aqueous solutions for which no experimental data are available.[9]

Computational Methods

The ab initio linear combination of the atomic orbitals−molecular orbitals method was used in this study. The calculations were carried out using the Gaussian 98[13] and Gaussian 03[14] computational chemistry programs. The geometry optimizations of all local minima have been performed at the B3LYP level of theory using the cc-PVDZ basis set without any symmetry restrictions. The characteristics of local minima were verified by establishing that the matrices of the energy second derivatives (Hessians) have no negative eigenvalues for all optimized structures.

The values of Gibbs free energy and corresponding equilibrium constants have been calculated using the standard formulas $\Delta G = \Delta H - T\Delta S$ and $K = e^{-\Delta G/RT}$, respectively, at room temperature (298 K). To estimate the ΔH values, the thermal corrections to enthalpy have been added to the corresponding energies.

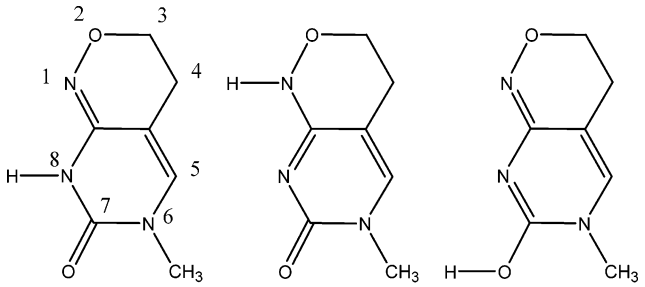

Figure 3. Structures and atom numbering of imino, amino, and enol tautomers of *N*-methyl-P.

The influence of water has been accounted for by using the polarizable continuum model[15] (PCM) of solvation as implemented in the Gaussian 03 program as well as the explicit inclusion of a limited amount of water molecules. The PCM of solvation has been applied to the structures of *N*-methyl-P (isolated and with four water molecules) with full geometry optimization at the B3LYP level of theory. All B3LYP/PCM optimizations of *N*-methyl-P have been performed using Gaussian 03. In all cases, thermal corrections to enthalpy and entropy values calculated at the B3LYP level of theory for the corresponding gas-phase tautomers (with one, two, three, and four water molecules) have been used in the estimation of the Gibbs free energies in aqueous solutions. Since all polar groups of the P, with which water molecules can interact, are covered with four water molecules, we assume that such complexes represent the structures with a completed first hydration shell.

The specific hydration by explicit water molecules has been performed in the following way. Initially, one water molecule was placed in different sites near the polar groups of the *N*-methyl-P molecule, and all possible structures were optimized. The second water molecule was added to all possible sites of the minimum-energy structure with one water molecule from

TABLE 1: Relative Gibbs Free Energies (kcal/mol) of *N*-methyl-P Tautomers in the Gas Phase, Equilibrium Constants, and the Derived Concentration Ratios

system	tautomers			K_{eq}	imino/amino
	imino	amino	enol	imino ↔ amino	concentration ratio
isolated	0.00	5.26	17.37	1.39×10^{-4}	7175:1
with 1 water molecule	0.00	4.33	12.79	6.70×10^{-4}	1493:1
with 2 water molecules	0.00	2.60	10.21	1.24×10^{-2}	81:1
with 3 water molecules	0.00	1.64	9.16	6.28×10^{-2}	16:1
with 4 water molecules	0.00	2.67	9.10	1.10×10^{-2}	91:1

TABLE 2: Calculated Charges on Selected Atoms and Dipole Moments in Imino and Amino Tautomers (see Figure 3 for Atom Numbering)

tautomer	atoms					dipole, D
	N1	O2	N8	H1/H8	O7	
isolated imino	−0.35	−0.21	−0.53	0.27	−0.52	4.81
isolated amino	−0.28	−0.36	−0.78	0.31	−0.56	5.60
imino·$1H_2O$	−0.22	−0.30	−0.51	0.28	−0.53	5.60
amino·$1H_2O$	−0.29	−0.27	−0.62	0.30	−0.54	6.11
imino·$2H_2O$	−0.27	−0.35	−0.45	0.30	−0.49	5.33
amino·$2H_2O$	−0.36	−0.27	−0.57	0.42	−0.55	5.82
imino·$3H_2O$	−0.32	−0.29	−0.47	0.38	−0.49	6.73
amino·$3H_2O$	−0.37	−0.24	−0.60	0.38	−0.52	8.20
imino·$4H_2O$	−0.25	−0.30	−0.49	0.34	−0.56	8.20
amino·$4H_2O$	−0.43	−0.14	−0.60	0.39	−0.49	9.76

the previous optimization. The same procedure has been repeated to find the lowest-energy structures with three and four water molecules. The results of this study reveal only the structures in which water molecule(s) occupy the site(s) with the highest attraction to *N*-methyl-P's polar groups (i.e., structures with the lowest Gibbs free energies).

The charges on the atoms were produced to fit to the electrostatic potential of the studied species at points selected according to the CHelp scheme as implemented in Gaussian.

Results and Discussion

First of all, we would like to point out that, in addition to the imino and amino tautomers of *N*-methyl-P depicted in Figure 2, the enol tautomeric structure should also be taken into account, since previous studies of DNA bases indicate a relatively high gas-phase population of such tautomers.[8a−h,m−q,9] Therefore, we have chosen all three possible tautomers of *N*-methyl-P (Figure 3). The results of the calculations are presented in Tables 1 and 2. Table 1 lists the values of the relative Gibbs free energies of the three isolated tautomers as well as tautomers with one, two, three, and four water molecules in the gas phase (structures are shown in Figures 4−7). The concentration ratios of different tautomeric forms calculated from the equilibrium constants are also shown. Table 2 depicts the values of the relative Gibbs free energies for the tautomers in aqueous solution along with the corresponding concentration ratios.

One may see that in the gas phase the stability of the enol form, as expected, is much lower than that of either of the other two tautomers of *N*-methyl-P. Thus, this tautomer is predicted to exist in very small undetectable amounts. The Gibbs free energy of formation of another *N*-methyl-P form, the amino tautomer, is much lower than that of the enol form. However, the energy difference with the imino form of more than 5 kcal/mol indicates that in the gas phase *N*-methyl-P will primarily exist in the form of an imino tautomer with an imino/amino concentration ratio of about 7175:1.

The Gibbs free energy differences change significantly after adding one or more water molecules. (The optimized geometries of complexes with one, two, three, and four water molecules are presented in Figures 4−7.) The interaction with polar water molecules stabilizes more the amino and enol tautomeric forms than the most stable gas-phase imino form. One may see that the relative Gibbs free energies for both amino and enol forms gradually decrease when one, two, or three water molecules are added to *N*-methyl-P. The addition of a fourth water molecule, however, stabilizes the imino form more than the amino form. Thus, the relative Gibbs free energy of the amino form with four water molecules increases compared to the structure with three water molecules reversing the trend. The concentration ratio of the imino and amino forms decreases from 7175:1 in the gas phase with no water molecules down to as little as 16:1 when solvated by three water molecules (but increases to 91:1 when the fourth water molecule is added). This indicates that the theoretical calculations correctly predict the trend and the extent of the effect of the polar surrounding (as represented by the water molecules) toward a greater stabilization of amino rather than imino tautomer.

The unexpected stabilization of the imino form by the fourth water molecule deserves closer attention. To explain the greater stabilization of the amino form rather than the imino form caused by the interaction with the first three molecules and the opposite effect of the fourth molecule, we have calculated the charges on the atoms in isolated and all hydrated imino and amino tautomers. The charges on the atoms that are the most important in hydrogen-bonding formation are presented in Table 2. The analysis of the charges on atoms interacting with water molecules clearly shows why the first three molecules stabilize the amino form more and why the effect of the next water molecule is opposite. For example, in isolated *N*-methyl-P, the charge on the N8 atom in the amino form is −0.78, while the charge on the N1 atom in the imino form is only −0.35. In addition, the hydrogen atoms that participate in hydrogen bonding with the first water molecule also have different charges, with the positive charge on H1 in the amino form being slightly higher than that on the H8 hydrogen in the imino form. Thus, the interaction with the first water molecule is greater for the amino form than the imino form. For the same reasons, the second and third water molecules interact greater with the amino form. On the other hand, the last water molecule stabilizes the imino form more. Again, while examining the charges on the O2 atoms to which the fourth water molecules are being added in the trihydrated species, one can see that the charge on the oxygen atom in the imino form is slightly greater than that in the amino form. This leads to a greater interaction of the water molecule with the imino form.

However, to correctly predict the true full effect of the polar surrounding, one would need to build a complete hydration shell around the molecules, which is very expensive computationally. An alternative to building a complete hydration shell with explicit water molecules is to use one of the continuum solvation models, for example, PCM,[15] which is used most frequently or a hybrid model which will take into account the influence of

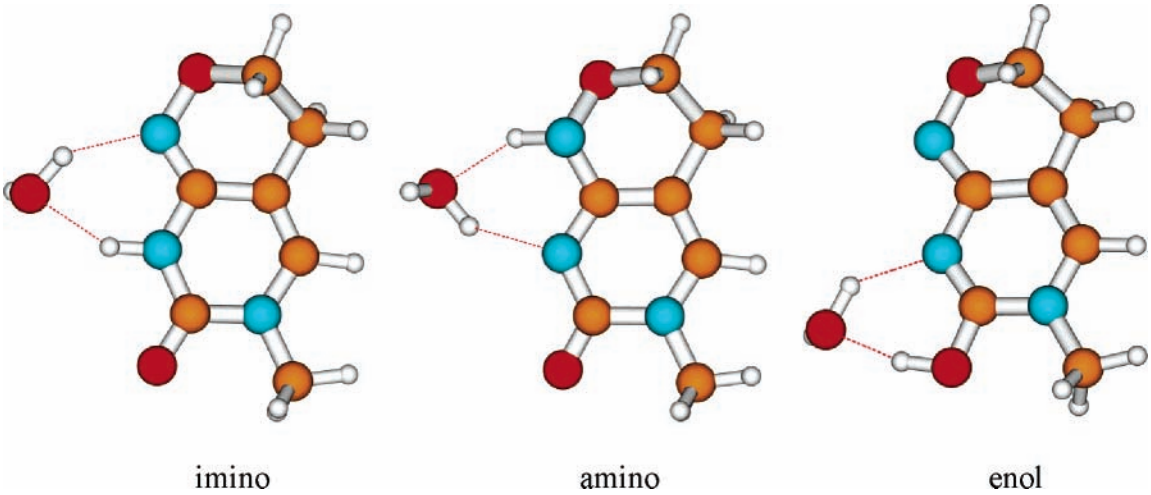

Figure 4. Optimized geometries of *N*-methyl-P tautomers with one water molecule in optimal positions.

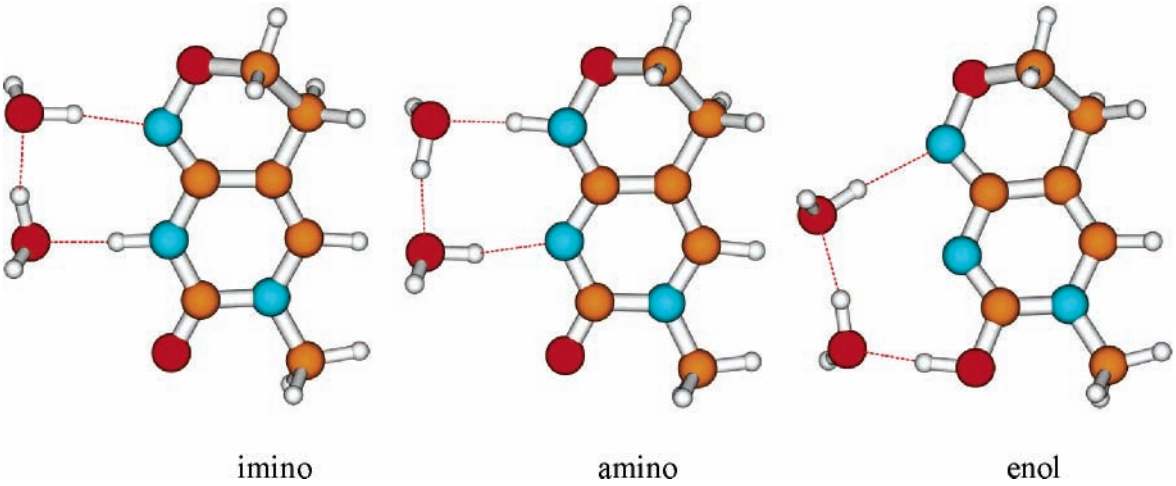

Figure 5. Optimized geometries of *N*-methyl-P tautomers with two water molecules.

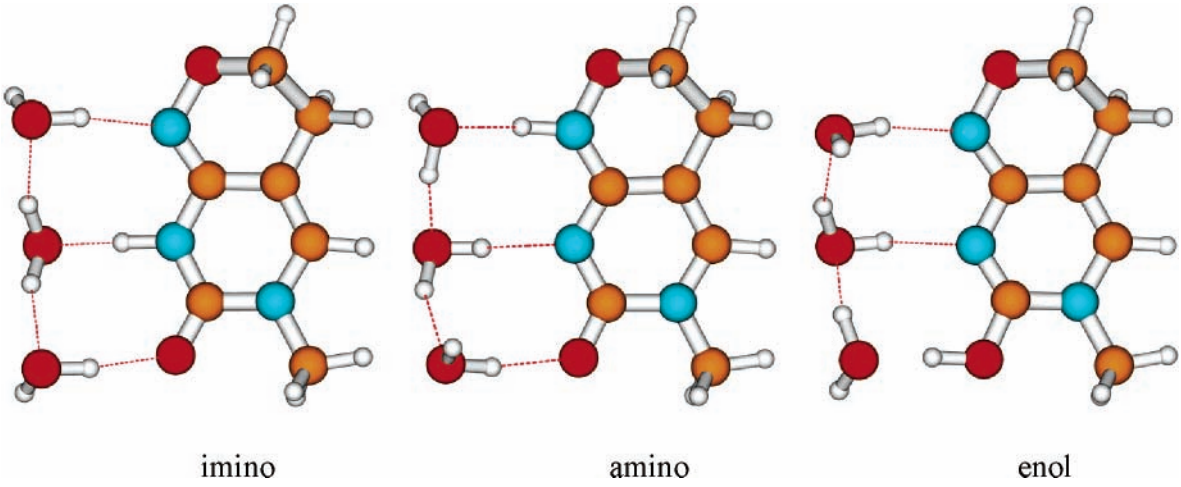

Figure 6. Optimized geometries of *N*-methyl-P tautomers with three water molecules.

explicitly included water molecules forming the first solvation shell and the influence of a continuum for the rest of the water bulk.

Since we estimate that the four water molecules comprise the first hydration shell of *N*-methyl-P, the PCM calculations have been performed for tautomers hydrated with four water molecules and, for comparison, also for the isolated species immersed in a dielectric continuum. Table 3 lists the relative Gibbs free energies of the tautomers of *N*-methyl-P which are isolated and hydrated with four water molecules, estimated within the PCM of solvation at the B3LYP level of theory (with full optimization). One can see that the optimizations of the isolated structures within the PCM reduce the gas-phase difference in energy between the imino and amino tautomers, leading to a ratio of 267:1. The reduction is partly due to the higher dipole moment of the amino form (see Table 2). Even though this is a significant reduction, it is not enough to reproduce the experimental results. On the other hand, the PCM

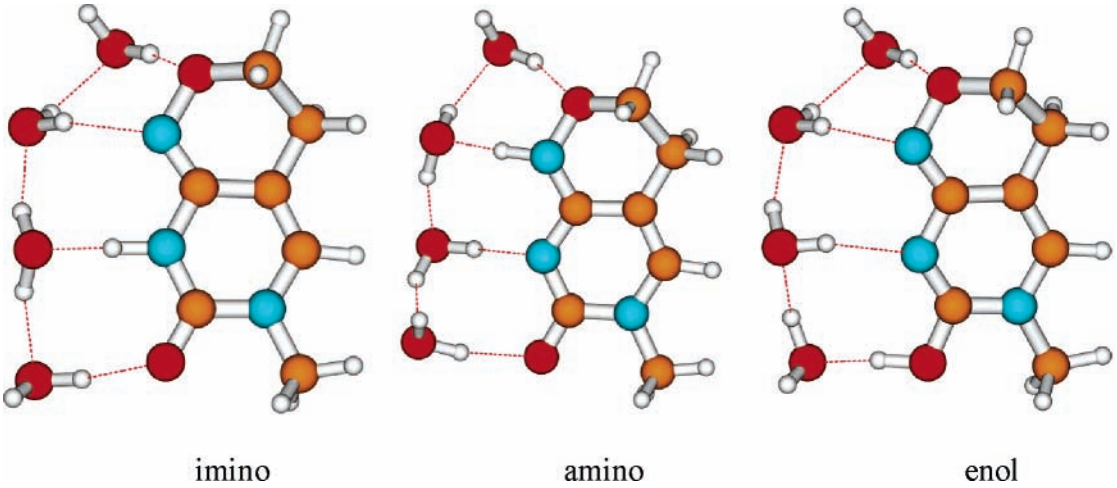

Figure 7. Optimized geometries of *N*-methyl-P tautomers with four water molecules.

TABLE 3: Relative Gibbs Free Energies (kcal/mol) of *N*-methyl-P Tautomers in Aqueous Solution (PCM), Equilibrium Constants, and the Derived Concentration Ratios

system	tautomers			K_{eq}	imino/amino
	imino	amino	enol	imino ↔ amino	concentration ratio
isolated	0.00	3.31	12.89	3.75×10^{-3}	267:1
with 4 water molecules	0.00	1.08	9.52	1.62×10^{-1}	6:1

coupled with the specific hydration accurately represents the experimental results. Thus, for example, the addition of four water molecules and optimization of the structures within the PCM decreases the ratio to just 6:1 ($\Delta G = 1.08$ kcal/mol), which is very close to the experimentally found ratio 11:1 ($\Delta G = 1.42$ kcal/mol).

The study has revealed that the concentration of the enol form remains very small even in the presence of a polar surrounding. However, the relative Gibbs free energy of the enol form decreases significantly in going from isolated to hydrated species. Thus, the results show that the relative stability of the enol form decreases from over 17 kcal/mol to around 9 kcal/mol. With the latter value, the concentration of the enol form should be over 10^6 times smaller than the concentration of the major tautomeric form. Even though such a small concentration cannot be detected experimentally, it may play a role in the spontaneous point mutations in DNA bases. Unfortunately, in an experimental study,[12] the authors did not take into account the possibility of the existence of a third tautomer. Consequently, the fixed enol tautomer was not synthesized; therefore, its absorption spectrum was not measured.

Though the current study addresses the tautomeric properties of the isolated tautomers of *N*-methyl-P, it also sheds light on the analogous properties of larger biopolymers. Our recent study[16] suggests that the tautomeric properties of all isolated DNA bases and anti conformers of 2′-deoxyribonucleotides are virtually the same. We believe that the same conclusion applies to *N*-methyl-P tautomers.

Conclusions

The results of this study provide the tautomeric equilibrium constants and concentration ratios for tautomers of *N*-methyl-P. The predicted properties are practically the same as those obtained experimentally. Thus, one can conclude that there are two independent sources (theoretical and experimental) that lend support to the proposal that the minor tautomeric forms of the natural bases may play an important role in substitution mutagenesis during DNA replication.

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Supporting Information Available: Complete refs 13 and 14. This material is available free of charge via the Internet at http://pubs.acs.org.

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