Quantum Mechanical Calculations of Nucleophilic Attack in the Pseudouridine Synthesis Reaction

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Abstract: *Ab initio* quantum mechanical calculations at the MP2 level were performed to model nucleophilic attack in the pseudouridine synthesis reaction. The energy profile along the reaction coordinate suggests that the C1′ attack by Asp may be the first step of the reaction, despite the fact that a COO^- is a relatively weak nucleophile. This result supports the new mechanism proposed by Huang et al. for this enzyme.[1] Our calculations also showed that nucleophilic attack by Asp on Cl′ was stabilized by the uracil ring and that a similar stabilizing effect could exist in other nucleotides.

I. Introduction

Pseudouridine (Ψ) is the most common modified nucleotide present in 93 modified bases identified in various RNAs.[2] Although its roles in biological systems are not fully understood, it exists in transfer RNA (tRNA), ribosomal RNA (rRNA), and small nuclear RNA (snRNA) and is present in all organisms ranging from prokaryotes to mammals. The unique carbon−carbon glycosyl bond has only been found in pseudouridine and its derivatives.

Pseudouridine Synthases (ΨS) catalyze the conversion of specific uridine residues in RNA into pseudouridine. Not much is known about its mechanism of action. A commonly proposed mechanism is as follows: the C6 carbon is first attacked by a cysteine residue, which serves as a nucleophile, followed by the cleavage of the carbon−nitrogen glycosyl bond. The uracil ring then processes a 180° flip (or 120° rotation), followed by the formation of the C5−C1′ bond to form the final product.[3,4] This mechanism is similar to the Michael addition type mechanism found in methyltransferases such as thymidylate synthase,[5] dUMP, and dCMP hydroxymethylases,[6] DNA (cytosine-5)-methyltransferases,[7,8] and tRNA (m5U54) methyltransferase.[9,10] In those cases, the nucleophile is the thiol from a cysteine residue of the enzyme. Attack at C6 of the pyrimidine ring forms the covalent cysteine intermediate, which results in activation at C5 for electrophilic attack. The attack is followed by an alkylation reaction at C5 of the pyrimidine ring. The difference is that in the methyltransferase case, the alkylation is intermolecular, while in the pseudouridine synthase case, it is intramolecular.

The above mechanism is consistent with most experimental evidence. Kammen et al. showed that tRNA Pseudouridine Synthase I (ΨSI) activity was inhibited by sulfhydryl reagents.[11] 5-FUra-RNA, which can form stable 5,6-dihydropyrimidine adducts with enzymes involving a methyltransferase mechanism, has been shown to be an inhibitor of ΨS.[12] However, no covalent intermediates have yet been detected as conclusive evidence in the 5-FUra-RNA interaction with TS. Recently, it also has been shown that a Cys residue is not conserved and, moreover, Cys is even not required for catalytic activity in the ΨS reaction.[1,4] The last two pieces of evidence clearly argue against the above sulfhydryl mechanism.

Huang et al. have proposed an alternative mechanism in which a conserved Aspartate serves as the nucleophile and the nucleophilic attack occurs at either the C6 or the Cl′ position as the first step in catalysis.[1] Although COO^- is known as a weak nucleophile, it has been found that aspartate or glutamate may serve as the catalytic nucleophile in glycosidases.[13−17] In tRNA guanine transglycosylase, a covalent reaction intermediate has been isolated and supports the fact that an aspartate residue can serve as the catalytic nucleophile to attack the Cl′ carbon.[18,19] This mechanism is very similar to the mechanism proposed by Huang et al.

High-level quantum mechanical calculations have been shown to be useful tools for studying reaction mechanisms and

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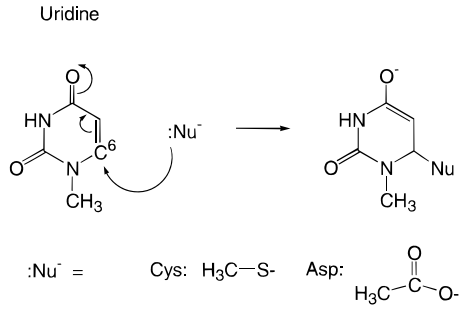

Figure 1. The reaction and the model molecules used in the C6 position attack. "Nu" is the nucleophile that can be a Cys (modeled by CH_3S-) or an Asp (modeled by CH_3COO-).

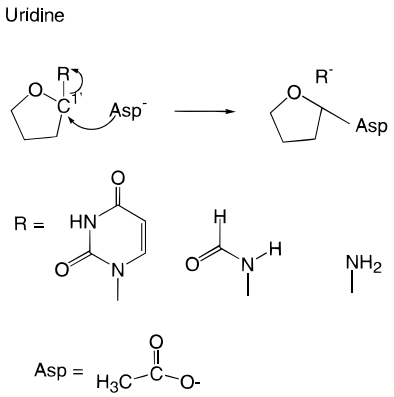

Figure 2. The reaction and the model molecules used in the Cl′ position attack. Three different R groups have been used to examine the effect of the uracil ring.

pathways. Thus, to examine the ΨS mechanism, we have performed high-level quantum mechanical calculations on model systems of this enzymatic reaction and have tried to analyze aspects of both the original mechanism of TS and the new mechanism proposed by Huang et al. The calculations we performed were to determine an energy profile along the reaction coordinate. From those data, we examined the energy barrier for the nucleophilic attack and the relative stabilities of reaction intermediates for different mechanisms. Because Cys is not conserved in all pseudouridine synthases, the most likely nucleophile should be the conserved Asp residue near the active site. Thus we first modeled the nucleophilic attack by Asp and by Cys at the C6 position. We also calculated the nucleophilic attack by Asp at the Cl′ position, as in the new proposed mechanism.

II. Methods

For the nucleophilic attack on the C6 position, we chose model molecules as shown in Figure 1. The energy profile along the reaction coordinate is obtained by constraining the distance between the nucleophile and the C6 atom of the uracil ring and calculating the MP2/6-31G+(d) single point energy using the HF/6-31+(d) optimized geometry. All geometric parameters were optimized except the constrained distance.

For the nucleophilic attack on the C1′ position, the model molecules are shown in Figure 2. The calculation protocol is the same as the calculations for the C6 position attack except the constrained distance is the distance between the nucleophile and the Cl′ atom of the sugar ring.

All calculations were done in the gas phase. Because there is no charge cancellation in the reaction and the active site is not solvent accessible, it should be a reasonable first step to model the reaction in the gas phase. All calculations are done on an SGI/Origin2000 workstation with 195 MHz CPU's and 256 MB memory. The Gaussian94 package was used.[20]

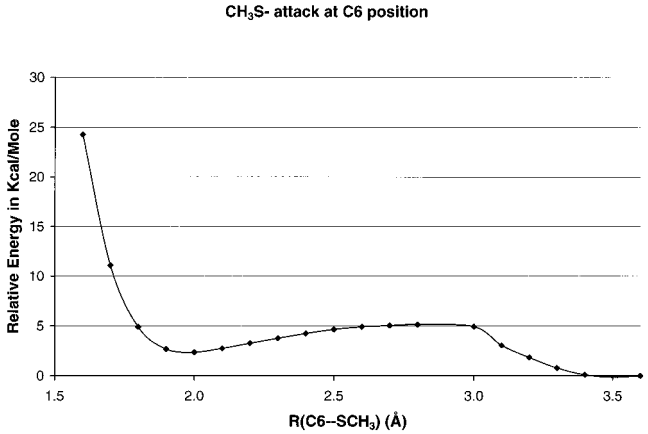

Figure 3. The energy profile for the C6 position attack by Cys (modeled by CH_3S-). The *x* axis is the distance between the nucleophile and the C6 atom (in angstroms). The *x* axis is the distance between the nucleophile and the C1′ atom (in angstroms). The *y* axis is the energy relative to $r = 3.6$ Å. The unit for energy is kcal/mol.

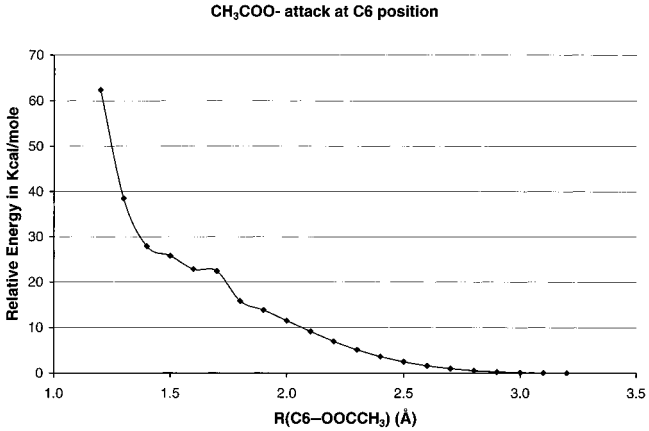

Figure 4. The energy profile for the C6 position attack by Asp (modeled by CH_3COO-). The *x* axis is the distance between the nucleophile and the C6 atom (in angstroms). The *y* axis is the energy relative to $r = 3.2$ Å. The unit for energy is kcal/mol.

III. Results

The energy profile along the reaction coordinate is shown in Figure 3 for the C6 position attack by Cys. The results show the attack by Cys has a local energy minimal at $r = \sim 1.9$ Å, which corresponds to the covalent reaction intermediate. The attack by Asp shown in Figure 4, however, does not have any minima along the reaction coordinate. Thus, Asp is a much weaker nucleophile than Cys and cannot form a stable intermediate during attack at the C6 position. Combined with the fact that Cys is not conserved and not required for the ΨS reaction, we can conclude that the C6 position attack by an Asp of ΨS on uridine is unlikely.

We also tested Asp attack at the C6 position of 5-F uracil. We found that the 5-F substitution slightly stabilized the adduct,

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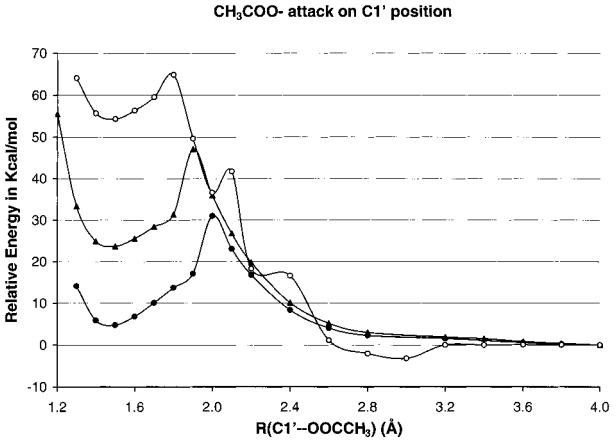

Figure 5. The energy profile for the Cl′ position attack for different leaving groups: filled circle, full uracil ring; filled triangle, −HCONH; open circle, $-NH_2-$. The *x* axis is the distance between the nucleophile and the C1′ atom (in angstroms). The *y* axis is the energy relative to $r = 4.0$ Å. The unit for energy is kcal/mol.

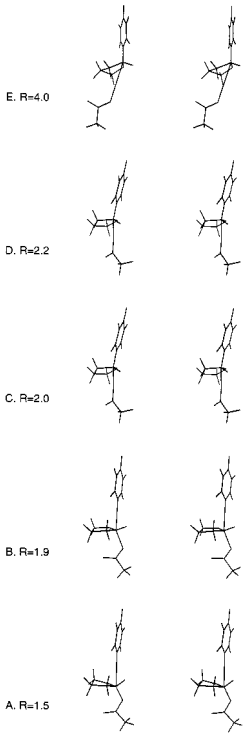

Figure 6. Stereoview of the geometries of different points along the reaction coordinate for the Asp attack at the Cl′ position. R is the distance between the Cl′ atom and the nucleophile.

leading to an energy increase relative to separate reactants of only ∼20 kcal/mol at an O−C6 distance of 1.5 Å, compared to ∼25 kcal/mol in Figure 4 at this distance. However, the basic shape and energies in the potential surface were quite similar to Figure 4 and very different from Figure 3.

The Cl′ position attack by Asp is shown in Figure 5. The results show that the attack by Asp has a covalent intermediate at $r = \sim 1.5$ Å, and the reaction has an energy barrier about 31 kcal/mol. The energy of the covalent intermediate is higher than the energy of the separate reactants by about 5 kcal/mol.

The geometries of different points along the reaction coordinate are shown in Figure 6. The distances between the Cl′ atom and the nucleophile for those geometries are $r = 1.5$, 1.9, 2.0, 2.2, and 4.0 Å and the C1′−N1 distances are 3.28, 3.00, 1.66, 1.56, and 1.50 Å, respectively. Thus the C1′−N1 bond is

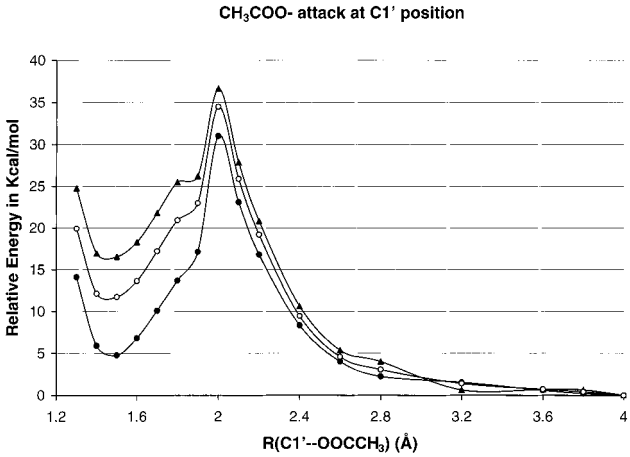

Figure 7. The energy profile for the C1′ position attack by Asp (modeled by CH_3COO-) in different dielectric environments using the COSMO model: filled circle, vacuum; filled triangle, high dielectric constant ($\epsilon = 78.4$); open circle: low dielectric constant ($\epsilon = 4.9$). The *x* axis is the distance between the nucleophile and the Cl′ atom (in angstroms). The *y* axis is the energy relative to $r = 4.0$ Å. The unit for energy is kcal/mol.

broken between $r = 1.9$ and 2.0 Å. The reaction intermediate, corresponding to $r = 1.5$Å, is an ion−molecule complex.

A rough estimate of the solvent effect was obtained using the COSMO model[21,22] in the Gaussian98 package.[23] We calculated solvation free energies at the MP2/631+G* level for the energy profiles of the Cl′ position attack by Asp using low ($\epsilon = 4.9$) and high ($\epsilon = 78.4$) dielectric constants. The results are shown in Figure 7 and reflect the fact that the charge distribution is more delocalized in the reaction intermediate than in the reactants. As a result, the energy barrier and the relative energy of reaction intermediate become higher when the solvation effect is considered. The barrier to Cl′ attack on uracil is raised to ∼35 kcal/mol and the reaction intermediate has an energy ∼12 kcal/mol higher than reactants in the low dielectric environment ($\epsilon = 4.9$) and they become ∼37 kcal/mol and ∼17 kcal/mol in the high dielectric environment ($\epsilon = 78.4$).

To examine the role of the uracil ring on the reaction pathway and energies, we also replaced the uracil ring by $-NH_2$ and −NHCOH and performed the same type of calculations. The results are also shown in Figure 5. For $-NH_2$ substitution, the energy barrier is ∼64 kcal/mol while the covalent intermediate is ∼54 kcal/mol higher than the reactants. For −NHCOH substitution, those numbers are ∼47 and ∼23 kcal/mol, respectively. From those calculation results, it is clear that the uracil ring stabilizes the covalent intermediate and reduces the energy barrier.

As noted above, in tRNA guanine transglycosylase, an aspartate residue serves as the catalytic nucleophile to attack

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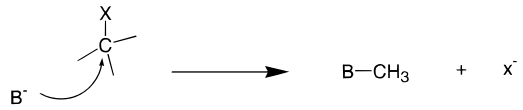

Figure 8. A typical nucleophilic reaction. B is the attacking base. X− is the leaving group.

Table 1. Relative Stability of Different Groups in Nucleophilic Reactions[a]

group, R	energy of CH_3-R	energy of R−	energy difference	rel stability (to NH_2)
NH_2	−95.20982862	−55.4760761	39.73375252	0
CONH	−207.9613817	−168.3107645	39.65061714	−52.13
A	−503.5534442	−463.9506058	39.60283833	−82.08
T	−490.5393282	−450.9432045	39.59612369	−86.29
C	−431.6457345	−392.029888	39.61584651	−73.93
G	−578.4274818	−538.8273395	39.60014233	−83.77
U	−451.5003201	−411.9082986	39.59202144	−88.87

[a] The unit for the first three columns is hartree while the unit for the relative stability is kcal/mol. The relative stability is defined as the energy difference (column 3) related to NH_2.

the Cl′ carbon.[18,19] This nucleophilic attack is almost identical with the Cl′ attack in pseudouridine synthase except the leaving group is a guanine, not a uracil. Our calculations show the uracil ring can stabilize the covalent intermediate and reduce the energy barrier for catalysis. Here we performed a set of calculations to examine the effect of different purine and pyrimidine rings. Consider a simple nucleophilic reaction as in Figure 8. We can compare the relative stability by the energy difference between CH_3−X and X− for different leaving groups. The following groups were chosen as leaving groups: $-NH_2$, −NHCOH, adenine, guanine, uracil, cytosine, and thymine. The relative stability for each group was calculated by comparing with the $-NH_2$ group. The results are listed in Table 1. It is clear that all rings, A, T, C, G, and U, are able to stabilize the nucleophilic attack by ∼80 kcal/mol when compared to the $-NH_2$ group. Interestingly, U is a better leaving group than the other bases, suggesting a possible mechanism for discrimination by the enzyme for U over the other bases, particularly over cytosine.

IV. Conclusion

We have performed ab initio quantum mechanical calculations for the first step of nucleophilic attack in pseudouridine synthases. We found that for the C6 position attack, Cys forms a much more stable adduct than Asp. Thus, Asp is an intrinsically unfavorable nucleophile for the attack at this position. Combined with the fact that the Cys residue is not conserved and is not required for catalytic activity, we suggest that the nucleophilic attack in pseudouridine synthase is not likely to occur at the C6 position. Our modeling on C1′ position attack showed that the Cl′ attack by Asp is stabilized by the uracil ring and a stable reaction intermediate is formed. Hence all our results support the mechanism proposed by Huang et al.,[1] which suggested that the C1′ attack by Asp could be the first step of the mechanism. Our calculations showed that the energy difference between the reaction intermediate and the reactants is ∼5 kcal/mol and the energy barrier is ∼31 kcal/mol for Asp attack at the Cl′ position. The energy barrier is still high compared with typical enzymatic reactions. If the solvent effect is included, the energy barrier is suggested to be further raised. However, the influence from the protein environment has not been included because there is no X-ray structure available. In other cases, it has been found that the electrostatic interactions can certainly stabilize ionic reaction intermediates by amounts appropriate to reduce the energy barriers to the level of typical enzymatic reactions.[24−28]

We have also calculated the relative stability of forming this reaction intermediate for different purine and pyrimidine rings and found the same type of stabilization exists for the A, T, C, G, and U rings. A similar mechanism has been found in tRNA guanine transglycosylase,[18,19] in which the nucleophilic attack is stabilized by the guanine ring. A similar type of mechanism could exist in other nucleophilic reactions.

In conclusion, our results suggest that the first step of the ΨS catalytic reaction does not involving the C6 attack by a cysteine residue as previously thought. Cl′ attack by Asp is found to be a possible pathway. There are certainly other plausible routes for the ΨS catalytic reaction. For example, Schröder et al. have shown that in the NADH-glycohydrolase system, the O4′ atom of the sugar ring can be protonated by a nearby neutral aspartic acid or glutamic acid residue, followed by an S_N1 reaction occurring at the Cl′ atom.[29] In this case, the aspartic acid or glutamic acid residues serve as a general acid rather than a nucleophile. A similar pathway could exist in the ΨS catalytic reaction. Until the X-ray structure is available for this enzyme, one cannot carry out the detailed theoretical studies required to evaluate how Asp is functioning as a nucleophile or, in its protonated form, donating a proton to the sugar oxygen to enable glycosyl bond cleavage. Also, the availability of the X-ray structure is necessary to model the subsequent steps of the ΨS reaction, including ring rotation and C−C bond formation.

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