Electrostatic Role of Phosphate 2485 in the Large Ribosomal Unit from *H. marismortui*

Katrin Spiegel and Paolo Carloni*

International School for Advanced Studies and INFM-DEMOCRITOS National Simulation Center, via Beirut 2-4, 34100 Trieste, Italy

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One of the phosphate groups present in the large ribosomal subunit of *Haloarcula marismortui* (P2485) is highly buried in the active site. Its interactions with the protonated forms of A2486 and of the substrate N-term moiety—two positive species that could be involved in the enzymatic mechanism—are here investigated by molecular simulation methods. Molecular dynamics calculations are first used to construct fully hydrated structural models of the active site in the free state and in complex with a model substrate. Density functional and electrostatic calculations based on the Poisson−Boltzmann equation based on these models suggest that the phosphate moiety significantly stabilizes the protonated form of the substrate and, moreover, that of A2486 by long-range electrostatic interactions. In contrast, our calculations provide no evidence for the previously proposed stabilization of A2486 via a charge-relay mechanism.

Introduction

The phosphate diester group is a fundamental building block of nucleic acids. This negatively charged moiety is in general stabilized by (usually water-mediated) electrostatic interactions with monovalent cations or by (usually direct) interactions with divalent cations.[1] Further stabilization is achieved by hydration: on average, each terminal oxygen atom forms hydrogen bond interactions with three water molecules, whereas each ester oxygen interacts with one water molecule.[2,3] (Criterion for the formation of H bonding: d(O−O) < 3.4 Å; minimal donor−hydrogen−acceptor angle of 90°.)

A notable exception to this common feature is found in the active site of the large ribosomal unit from *Haloarcula marismortui*,[4−6] which catalyzes the formation of a peptide bond between an aminoacyl-tRNA group and the nascent peptide chain.[5,9]

Experimental structural information, available for the free state as well as for complexes with substrate analogues or inhibitors, reveals that the phosphate group belonging to adenosine 2485 (P2485 hereafter) is buried in the structure and its charge is not fully screened by the chemical environment. Specifically, the X-ray structure of the free state at 2.4-Å resolution[4] shows that the closest counterions from P2485 are (i) a K^+ ion, which is located 10 Å from the P2485 phosphorus atom. The ion is coordinated by RNA neutral groups (Figure 1 A). (ii) a Mg^{2+} ion, which although its distance to P2485 is shorter (d(Mg−P) = 6 Å) is fully screened by the macromolecular scaffold, as it is coordinated by C2533, C2534, and A2483 phosphate groups. The structure further shows that this phosphate group is also poorly hydrated, as it interacts with three water molecules directly (Figure 1). These peculiar structural properties of P2485 are present in a variety of complexes with antibiotics,[7,8] in which these ligands are located at the exit channel of the subunit (structures solved at 3 Å resolution−pdb entries 1K9M, 1K8A, and 1KD1−and 3.2 Å resolution−pdb entry 1M1K[7]). They could also be present in complexes with the substrate analogue puromycin, although in the structure at 3 Å this issue cannot be addressed, as water molecules and counterions could not be identified.[4] While submitting this paper, a structure at 2.8-Å resolution of the adduct with the substrate mimic CCA-Phe-caproic acid-biotin[8] confirmed the peculiar characteristics of this phosphate group in this complex.[8]

The issue addressed here is whether the presence of this partially unscreened charge could play a role in the stabilization of two positively charged species that might be present during the enzymatic reaction cycle. The first species is the protonated form of one of the reactants, that is, the incoming aminoacyl-tRNA whose N term might be expected to be protonated[8,10−12] ($[S-H]^+$ hereafter). The second species is the protonated form of a nucleobase in the active site, A2486 ($[A-H]^+$ hereafter).[9] This in turn could stabilize the negatively charged transition state of the ribosomal reaction.[5] $[A-H]^+$ stabilization has been proposed to arise either through a charge relay mechanism (Chart 1) or by electrostatic interactions.[5] However, although the *existence* of the $[A-H]^+$ species has been inferred by titration experiments on the isoenzyme from *E. coli*,[12] it must be pointed out that the *relevance* of this species to the enzymatic function has been seriously questioned.[13−15]

Here we used computational methods to investigate the role of P2485 in the stabilization of these cationic species. First, we use molecular dynamics (MD) model calculations to provide the hydration pattern at the active site in complex with the model substrate puromycin as well as an educated guess of the location of water molecules not yet detected in the free form. Then, the nature of the $[S-H]^+$− and $[A-H]^+$−P2485 interactions is investigated in these structural models by density functional and Poisson−Boltzmann (PB) electrostatic calculations. The calculations suggest that both protonated species are indeed significantly stabilized by electrostatic interactions in the low-dielectric medium present in the active site. In contrast, they provide no evidence for the postulated charge relay mechanism-based stabilization of $[A-H]^+$(Chart 1).[5]

* Corresponding author. E-mail: carloni@sissa.it. Tel. +39-040 3787 407. Fax: +39-040 3787 508.

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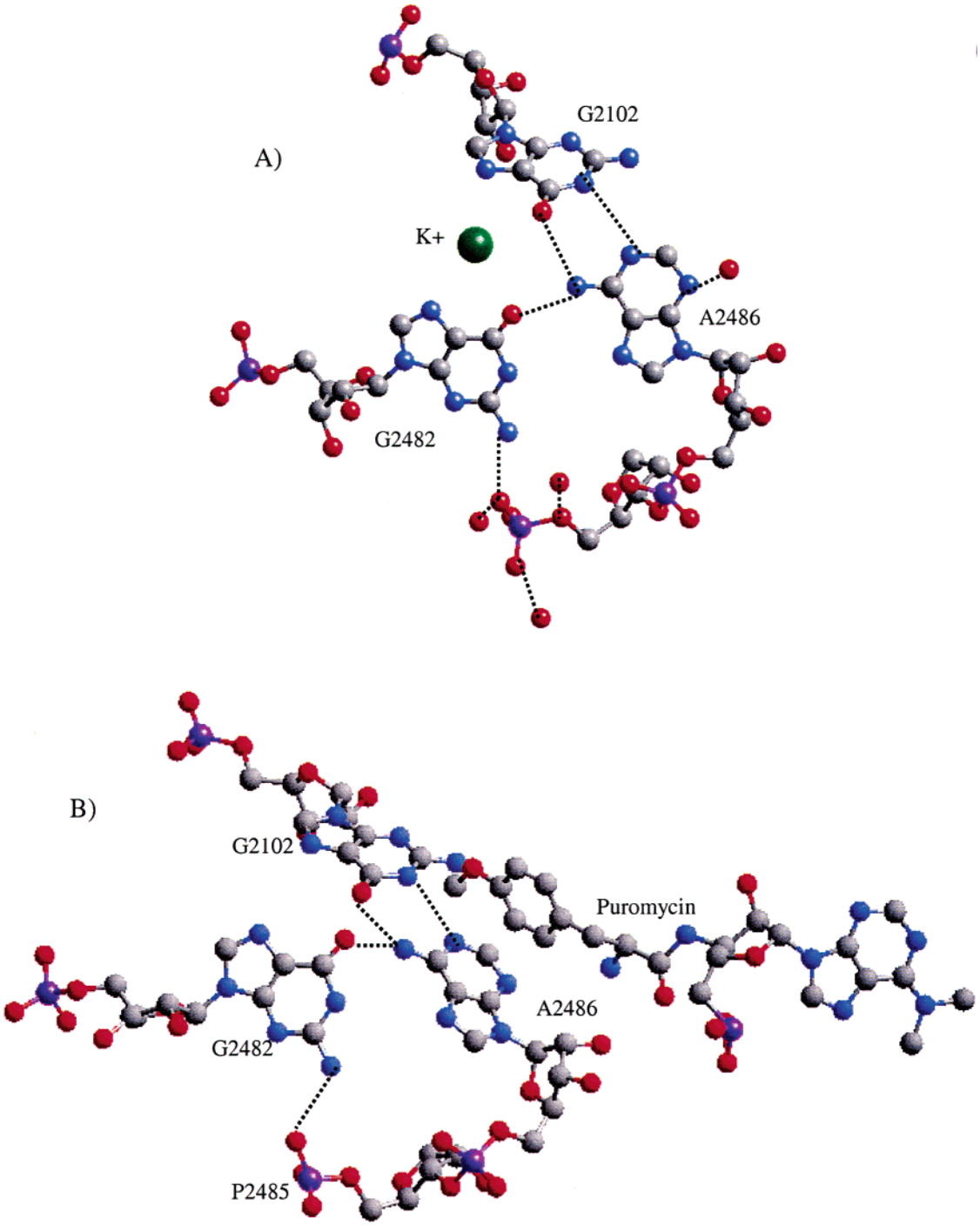


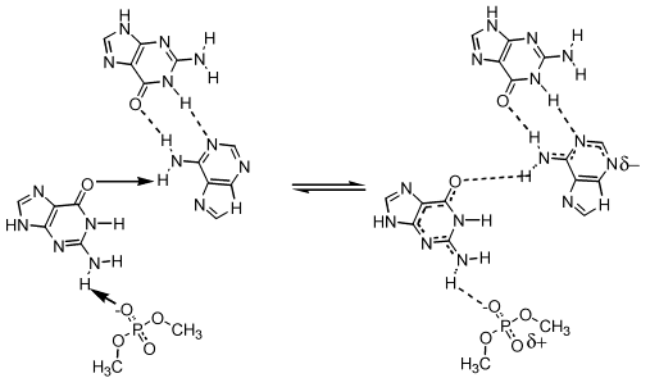
Figure 1. X-ray structures of the active sites of the large *H. marismortui* in the free state[4] and in complex with puromycin.[4,41] The fully conserved[5] A2486 group forms hydrogen bond interactions with G2102 and G2482. The latter also forms hydrogen bond interactions with P2485. (A) Free state: water molecules and the potassium ion are detected only in this state. The K^+ ion is coordinated by O6 and N7 of G2482 and to O6 of G2102.[4,5] (B) Complex with the puromycin substrate analogue. This is made up of 13 base pairs and (mimicking the acceptor stem of $tRNA^{tyr}$) linked to puromycin (by a phosphodiester bond),[5] which is an analogue of the tyrosyl group attached to the tRNA. Only the puromycin moiety is shown for clarity. The H-bond pattern involving the RNA groups at the active site is analogous to the free state. N3(A2486) is located 3.8 Å from the puromycin amino atom.

Methods

MD Calculations. Our models included the ribosome groups within 20 Å of N3 of A2486 (N3(A2486) hereafter) in the large ribosomal subunit of *H. marismortui* (pdb entry 1JJ2) in the free state and in complex with an aminoacyl-tRNA substrate analogue (pdb entry 1FG0).[16−18]

The model of the free enzyme also included 230 ordered water molecules and several counterions (1 potassium ion, 9 magnesium ions, and 7 sodium ions). In contrast, in the complexed state examined here, water and counterions are not detected, possibly because of the lower resolution of the structure. Because (i) the crystal structure of the complex has been obtained under the same conditions as those of the free enzyme[4,5] and (ii) the structures of the complexed and of the free states' active sites are very similar (rmsd lower than 1 Å), an educated, initial guess of the locations of the same counterions as those in the free enzyme can be obtained by fitting the residues within 5 Å of the free form with the corresponding

CHART 1



residues of the complex. The systems were immersed in a sphere of Monte Carlo waters[19] centered on N3(A2486),[19] taking care to exclude water molecules located in positions that would have been occupied by macromolecule atoms not included in the model. The radius of the sphere was 38 Å, resulting in an ~10 Å layer around the explicit RNA.

A model of N9-methyladenine in water was also considered. The molecule was solvated in a box of 15.1-, 13.3-, and 13.3-Å edge lengths, containing 74 water molecules.

The free state, the complexed state, and N9-methyladenine models consisted of 16 551, 16 236, and 237 atoms.

The TIP3P[19] parameters for the water molecules and the AMBER parm98 force field[20,21] parameters for the RNA residues and counterions were used, respectively. Approximately 800 dummy atoms, located in the positions of the macromolecule scaffold not included in the model but accessible to the solvent, were included so as to prevent the water molecules from occupying the space occupied by the macromolecule. These dummy atoms possessed the same van der Waals parameters as TIP3P oxygen and zero charge.

The three systems underwent 200-ps MD simulations using the SANDER module of the AMBER suite of programs.[22] Electrostatic interactions were either evaluated explicitly for all of the pairs (for the free and complexed state) or with the particle mesh Ewald (PME)[23,24] method (for N9-mehtyladenine in water).[21] A cutoff of 10 Å was used for the van der Waals interactions. A time step of 1 fs was applied. Simulations at 300 K were achieved by coupling the systems to a Berendsen thermostat.[25] A constraint force of 8 kcal/(mol Å^2) was applied to RNA during MD simulation. In this way, the solutes were kept close to their X-ray structure during the dynamics (final rmsd: 0.3 Å for both structures). For the complexed structure, the constraint on the substrate was released after 80 ps of simulation. The initial distance between the NH_3 group of the substrate and N3(A2486) decreased from 3.8 to 3 Å, with the formation of a hydrogen bond between these two groups.

DFT Calculations. The structural models were extracted from the final MD simulations reported above. **A**−**D** represented the free state (Figure 2). **A** included P2485, A2486 (modeled as N9-methyladenine); G2482 (modeled as a guanine residue), the moiety of G2102 H-bonding to A2486, (modeled as aldehyde); the K^+ ion; and two water molecules—WAT1, which interacts with P2485 and it is located in the region between P2485 and A2486, and WAT2, which forms an H-bond interaction with O5′ of P2485. In **B**, **C**, and **D**, P2485, G2482 and A2486, and the K^+ ion were absent, respectively. Models **F**−**I** represented the complexed state (Figure 3). **F** contained all of the groups present in **A** except that the model substrate *N*-methyl-α-aminobutanamide ($C_5N_2OH_{12}$) replaced water molecule WAT2 (Figure 3). The model substrate was obtained by replacing the tyrosyl side chain and the adenosyl group of puromycin by methyl groups (Figure 3). DFT calculations (see below) showed that the protomer in which the substrate N-term group was protonated (**F**, Figure 3) was more stable than that in which N(A2486) was protonated (Figure 4SI, Supporting Information) and was the only one considered here. In **G**, **H**, and **I**, the phosphate group, the guanine and the aldehyde moiety, and the K^+ ion were absent, respectively (Figure 3). The total charge for models **A**, **B**, **F**, and **G** was +1. That for models **C**, **D**, **H**, and **I** was 0.

The model of *neutral* N9-methyladenine in water was the final MD structure (**E**, Figure 2SI), and periodic boundary conditions were applied. That of *protonated* N9-mehtyladenine included only the first and second solvation shells (26 water molecules). Isolated molecule conditions[26] were applied.

Our quantum chemical calculations were carried out within the framework of density functional theory using the CPMD program.[27] The basis set consisted of plane waves up to an energy cutoff of 70 Ry. Core/valence electron interactions were described using normconserving pseudopotentials of the Martin Trouiller type.[28] The gradient-corrected Becke exchange functional and the Lee−Yang−Parr correlation functional (BLYP)[24,29] were used. The geometry of all models **A**−**I** were optimized by DFT-based molecular dynamics[30] slowly heating the system up to 300 K[31] and then performing at least 200 steps of annealing.[32] During the entire dynamics run, the positions of the peripheral atoms of the models were kept fixed (Figure 3SI). These constraints were meant to mimic the presence of the ribosomal scaffold, which is not included in the model. The polarity of the bonds ($\Delta\chi$) and the ESP charges were calculated from the Wannier centers[33,34] and from the electrostatic potential[35] following established procedures.

Electrostatics. The models of the free and complexed states were based on our final MD structures. Water and counterions were not explicitly included. Test calculations in which the counterions were explicitly included provided similar interaction free energies as calculations with implicit ionic strength.

The linear Poisson−Boltzmann equation was solved by a finite difference method using the DelPhi program.[36] The AMBER[22] partial atomic charges were used, and focusing was applied in order to have at least 3 grid points per Å. This turned out to be the minimal resolution required to reach converging results.

Results

MD Simulations. These calculations are used to provide a fully hydrated structural model of the active site of the large ribosomal subunit of *H. marismortui* in the free state and in complex with a model substrate (puromycin).

P2485 turns out to interact at most with three water molecules, as already shown in the X-ray structure of the free form.[4] (Water molecules within 3.4 Å of H-bond donors were here assumed to form H bonds.) Among the other 75 phosphates included in the model, 71 are fully hydrated, interacting on average with 6−8 water molecules (Figure 4) and usually also with counterions. Only four phosphates (P632, P2534 for P2608, and P2532) are as poorly hydrated as P2485 (Figure 4). However, the first three groups interact either with a magnesium ion or a potassium ion, and they are located at least 16 Å from A2486, to be compared to the distance between P2485 and N3(A2486) (10 Å). The last one is located as far as 18 Å from A2486. The remaining phosphate groups are fully hydrated, interacting on

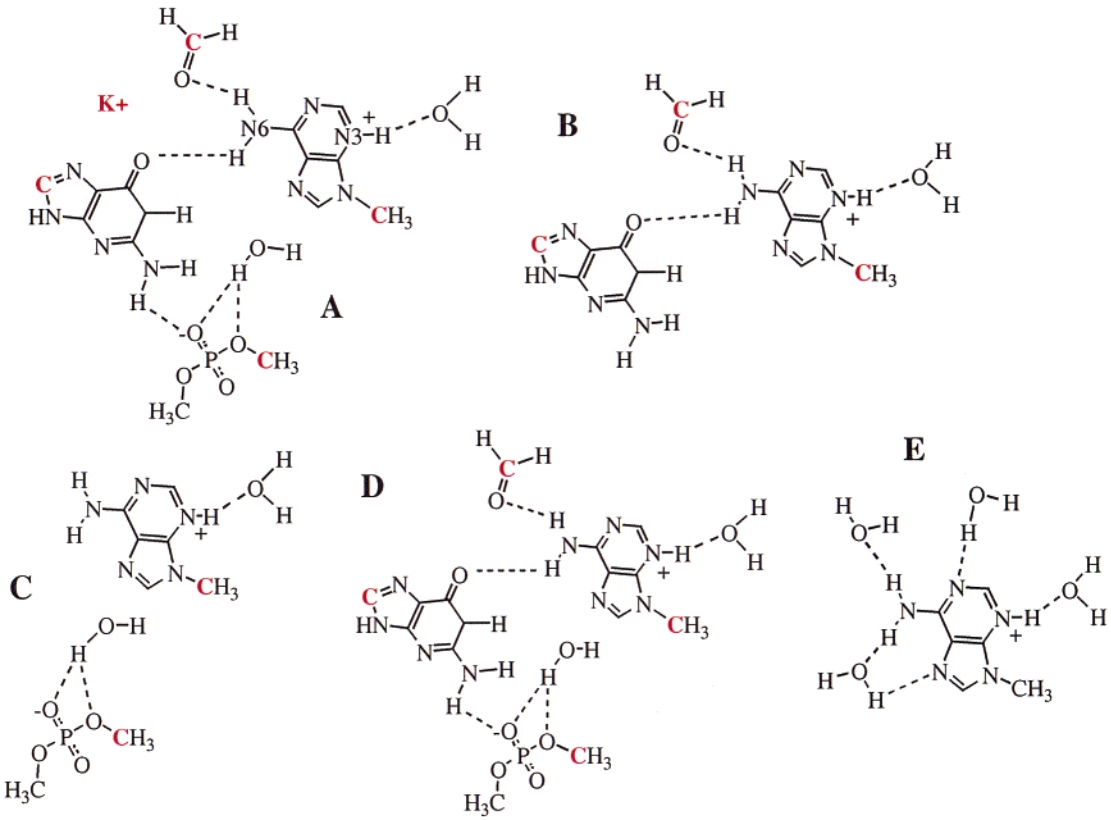


Figure 2. **A**−**D**: Quantum chemical models used for the free state based on MD calculations carried out here. Notice that A2486 is protonated and that two water molecules are present. WAT1 was already detected in the X-ray structure and forms H bonds to O3′, whereas WAT2, which was taken from an MD snapshot, forms a hydrogen bond to A2486 and it is part of the active-site channel. **E**: Model of N9-methyladenine in water. Only the waters interacting with the nucleobase are shown for clarity. Twenty-six water molecules were actually included in the calculation. The atoms subjected to position constraints are depicted in red.

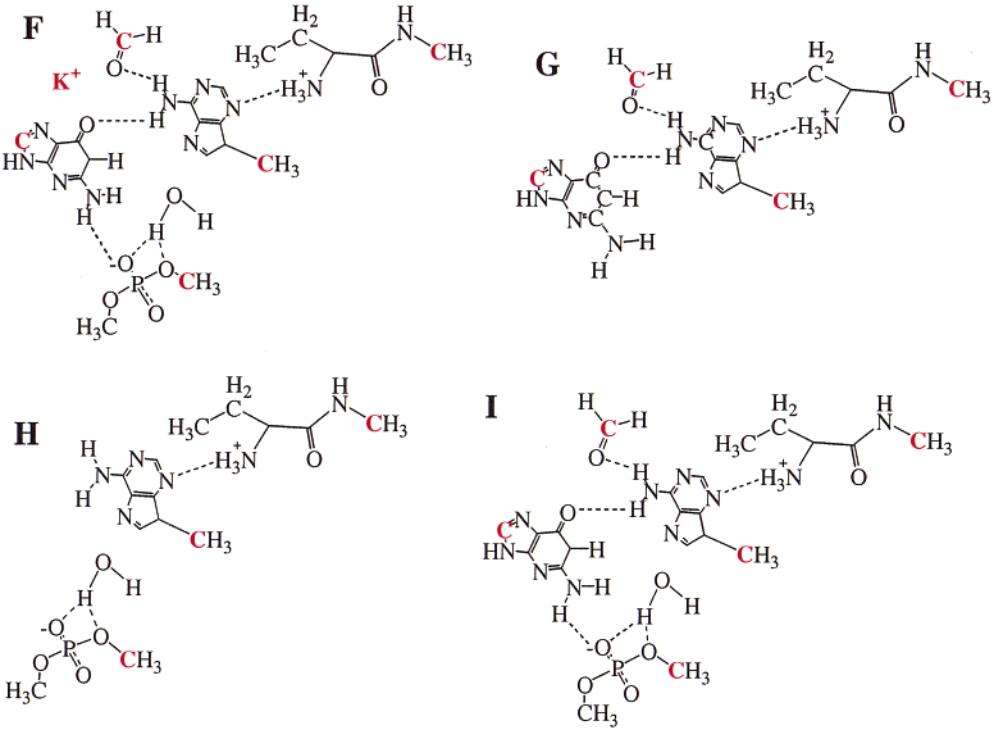


Figure 3. **F**−**I**: Quantum chemical models used for the complexed form based on MD calculations performed here. The substrate is protonated, and a water molecule is present. The atoms subjected to position constraints are depicted in red.

average with 6−8 water molecules (Figure 4), and they usually also interact with counterions.

In the complex with a model substrate (Figure 1B), P2485 also interacts with three waters, as in several other complexes with inhibitors.[6−8] The phosphate groups as poorly hydrated as P2485 (P2534, P2539, P2541, and P2608) are located at least

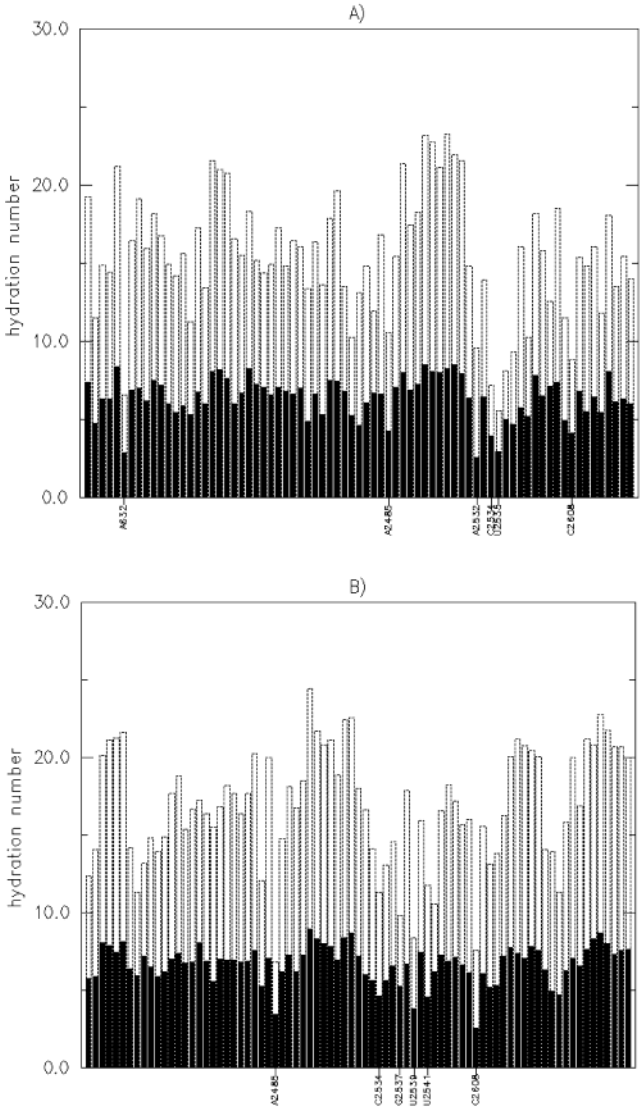


Figure 4. Number of water molecules surrounding the phosphate groups located within 20 Å from the N3(A2486) atom in the large ribosomal subunit of *H. marismortui*. The black and white histograms refer to those water molecules located within 3.4 and 5.0 Å for any phosphate oxygen atom moieties within a distance of 20 Å of A2486 for **(A)** the free form and **(B)** the complexed form.

14 Å from NH_3^+ (Table 1SI), whereas P2485 is located at 12 Å from the amine group.

Thus, our MD calculations suggest that P2485 is the most important phosphate group for the stabilization of the $[A-H]^+$ form (in the free state) and $[S-H]^+$ (in the complex with the substrate). The nature of these interactions is investigated in the next section by density functional theory (DFT) and electrostatic calculations based on the Poisson−Boltzmann calculations.

DFT Calculations. A comparison of the proton affinity of A2486 and of the model substrate in models of increasing complexity (Figures 2 and 3) is here used to estimate the stabilization provided by P2485. In the *free* form, the stabilization of $[A-H]^+$ is significant (Table 1). P2485 is the group that contributes most to the stabilization of $[A-H]^+$, despite the relatively large distance between the two moieties. In contrast, an analysis of the electronic structure of the complexes suggests that the proposed charge relay mechanism (Chart 1), in which the negative charge of P2485 is partially transferred to N3(A2486) through the H-bond pattern, is not operative.[5]

TABLE 1: Selected Properties of the Free State as Obtained by DFT Calculations[a]

			$\Delta\Delta H$ [kcal/mol]		
model	**A**	**B**	**C**	**D**	**E**
$\Delta\Delta H$ [kcal/mol]	0	34	−11	−40	
			ESP partial atomic charges		
N6 A2486	−0.649	−0.481	−0.642	−0.522	−0.596
N3(A2486)	−0.287	−0.287	−0.354	−0.310	−0.199
A2486	0.751	0.816	0.719	0.765	0.908
P2485	−0.594		−0.702	−0.834	
			$\Delta\Delta\chi$(N−H)A2486		
N6-H61	0.005	−0.014	0.004	−0.002	0
N6-H62	−0.001	−0.016	0.006	−0.016	0
N3-H3	0.010	0.016	0.017	0.014	0

[a] $\Delta\Delta H$ represents the protonation affinities relative to **A** [kcal/mol]. ESP charges are electrostatic potential-derived charges[35,42] expressed in [*e*]. $\Delta\Delta\chi$ represents the differences in the Pauling electronegativity[43] of chemical bonds. Here, negative (positive) values of $\Delta\Delta\chi$ indicate a stronger (weaker) polarization of the N−H bond in the active-site model than in water.

TABLE 2: Protonation Affinities ($\Delta\Delta H$) Relative to Model F

model	**F**	**G**	**H**	**I**	**A**
$\Delta\Delta H$ [kcal/mol]	0	∼0	−39	−37	−37

Indeed, the polarization of the NH and OH bonds involved in this network, here estimated in terms of changes in Pauling electronegativity $\Delta\chi$ upon formation of the complex, is very local in nature (Table 1, Table 3SI). In addition, A2486 H-bond donor and acceptor groups appear to be either equally or less polarized than in water solution, as suggested by the change in $\Delta\chi$ on passing from the complex to water solution (i.e., $\Delta\Delta\chi$ values close to zero or positive).[34,37,38]

In the *complexed* form, the effect of P2485 on the stabilization of $[S-H]^+$ is much less pronounced than that of P2485 on $[A-H]^+$(Table 2). This might be due to the larger distance between the two moieties (*d*P(P2485)−N(substrate) = 13.1 Å) and/or to a higher degree of hydration of $[S-H]^+$, which is located in the fully solvated active-site channel. In particular, the substrate NH_3^+ group forms on average two hydrogen bonds with two water molecules and with N3(A2486).

Role of the K^+ Ion in the Active Site. The potassium ion appears to be very important for stabilizing the negatively charged phosphate group in our models. Indeed, it affects the charge distribution of the phosphate moiety, as estimated with the ESP charges (Table 1).[4] (A complete list of all ESP charges is presented in Table 2SI.) Furthermore, the presence of this ion leads to a significant destabilization of $[S-H]^+$, as can be seen by comparing the proton affinities of **A** and **D** (free state) and **F** and **I** (complexed state). This is in particular the case of the adduct with the substrate: indeed, the calculated proton affinity of the structure without the K^+ ion (model **I**) is larger than the corresponding value for the free enzyme (model **D**). However, the actual effect of this ion is expected to be significantly smaller, as its charge is expected to be screened by an additional phosphate group of U2539, which is not included in the models.

Role of the Dielectric Medium. The DFT calculations are done in vacuum. To estimate the dependence of electrostatics from the dielectric properties of the medium at a qualitative level, the electrostatic interactions are solved at increasing values of the dielectric constant using the Poisson−Boltzmann equation.

At low dielectric constant values (5−10), as assumed for the active site of the ribozyme,[36] the electrostatic interaction free

TABLE 3: $[A-H]^+$− and $[S-H]^+$−P2485 Electrostatic Binding Free Energies for the Free and Complexed Forms, as Calculated Using the Poisson−Boltzmann Equation[a]

	$\Delta G_{binding}$ [kT]			
dielectric constant of the solvent	5	10	20	80
$[A-H]^+$	4.09	3.03	2.26	1.33
$[S-H]^+$	1.60	1.04	0.63	0.25

[a] The energies for different dielectric media are reported. The dielectric constant of the solute has been set to 2.

energy between P2485 and $[A-H]^+$ is significant (Table 3). At larger values, such as those assumed for duplex DNA/RNA in aqueous solution ($\epsilon = 20-30$ in the minor groove and $\epsilon = 40-60$ in the major groove[36]), the interaction free energies are small and do not differ largely from those in water (Table 3). Consistently with the DFT calculations, P2485/$[S-H]^+$ electrostatic interactions are less favorable than those between P2485 and $[A-H]^+$. At low dielectric constants (5−40), $\Delta G_{b[A-H]^+}$ and $\Delta G_{b[S-H]^+}$ differ by a factor of 3. Thus, $[A-H]^+$/P2485 interactions are larger than $[S-H]^+$/P2485 interactions.

Discussion

We have presented a theoretical study of the structural, energetic, and electronic properties of the catalytic site of the large ribosomal unit from *H. marismortui*. The calculations have been based on the X-ray structure of the large ribosomal subunit of *H. marismortui* in its free state[4] and in complex with a substrate analogue derived from puromycin,[5] which is structurally and chemically very similar to the aminoacyl-tRNA substrate.[4] MD calculations have been used to construct fully hydrated structural models of the active site involving two cationic species, which might be involved in the enzymatic reaction. The first is the protonated form of A2486 $[A-H]^+$, which could be involved in general acid/base catalysis or could stabilize the negatively charged transition.[5] However, the existence of such a species is the object of serious debate.[13−15,39,40] The second species is the protonated form of the substrate N-term ($[S-H]^+$ Figure 1B) that corresponds to the N-term of the substrate, the incoming aminoacyl-tRNA.

The MD calculations confirm that in the free form P2485 interacts on average with only three waters, as already seen in the X-ray structure, whereas most of the other phosphate groups are fully hydrated, interacting on average with six to eight water molecules. The same picture emerges in the complexed form, consistent with what has been observed in a variety of X-ray structures of complexes with different inhibitors.[6−8] In both the free and complexed states, only a few other phosphates groups are as highly dehydrated as P2485 at the active site. These groups are located farther than P2485 from the $[S-H]^+$ and $[A-H]^+$ species, and in most cases, they have water-mediated or direct interactions with counterions. Thus, P2485 emerges as the phosphate group that plays the most significant role in the stabilization of the $[S-H]^+$ and $[A-H]^+$ species.

DFT calculations have provided a description of the nature of the interactions between the cationic species and their interacting groups. $[S-H]^+$ and, moreover, $[A-H]^+$ species are stabilized significantly by long-range electrostatic interactions with P2485 (Tables 1 and 2). In contrast, our DFT calculations do not support the postulated charge-relay mechanisms (Chart 1) involving the G2482 and G2102 nucleobases[5] (Table 1). Because of the relative small size of the models used, the values reported above represent trends, and they cannot be considered quantitatively.

Our calculations based on the Poisson−Boltzmann equation further suggest that, although P2485 is located several angstroms from the two cations, it does effectively stabilize P2485 in the presence of the low-dielectric medium in the active site of the ribosome. Indeed, the stabilization would be much less effective in standard A- and B-RNA structures (Table 3), where the dielectric constant is much higher. Thus, P2485 might assist in steering the substrate into a favorable position for the peptidyl transferase reaction in which the amino terminus of the incoming aminoacyl-tRNA attacks the carbonyl carbon belonging to the last amino acid of the nascent peptide chain.

A comment is in order for the reliability of this structural information for constructing our model of the free state. Indeed, it has been suggested[13] that the conformation possessing the $[A-H]^+$ species in active ribosomes is different from the structural models that can be constructed on the basis of the X-ray structures.[13,15,39] Former titration experiments on the isoenzyme from *E. coli* have shown that the pK_a of A2486[12] increases by ∼6.5 units relative to the value in water. On the basis of the X-ray structure, it was believed that A2486 was protonated at the N3 atom since N1 form a hydrogen bond to G2102 (Figure 1). Subsequent experiments[39] cast doubt on this hypothesis, arguing that a pH-dependent structural rearrangement could change the solvent accessibility of N1. Very recent data has shown that the crystals of the large ribosomal unit from *H. marismortui* are fully active,[6] suggesting that the X-ray structure used here might provide a reliable model for the $[A-H]^+$ species. Furthermore, our electrostatic PB calculations suggest that the stabilization is not dependent on the fine details of the structure: our findings are expected not to be changed at the qualitative level when geometries of the active site that are significantly different from those that emerge from the X-ray structure are used.[13]

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Supporting Information Available: Structural properties of the MD models and selected electronic properties of complexes **A**−**D**. A comparison between the X-ray structure (green) and that obtained by DFT calculations (blue) of model **A** as well as a cartoon of our structural model of N9-methyladenine in water solution. Constraints applied to the DFT models. DFT model F1. This material is available free of charge via the Internet at http://pubs.acs.org.

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