Molecular Dynamics and Free Energy Perturbation Calculations on the Mutation of Tyrosine 45 to Tryptophan in Ribonuclease T_1

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The mutation of tyrosine 45 to tryptophan in ribonuclease T_1 enhances nucleolytic and binding abilities to its ligands [S. Nishikawa *et al.*, *Eur. J. Biochem.*, 173, 389 (1988)]. To examine the energetic and structural influence of this mutation in ribonuclease T_1, we made molecular dynamics and free energy perturbation calculations by converting the wild-type enzyme to the mutant one. The calculated difference of free energy changes following binding to 2′-guanylic acid (2′-GMP) molecule well agreed with experimental value. Between the mutant–2′-GMP complex and the wild-type–2′-GMP complex, subtle structural changes in stacking and hydrogen bonding interactions were found. Enhanced activity was almost entirely attributable to increased hydrophobic interaction between the ligand and the mutant enzyme. In the vicinity of ligand binding site, distributed water molecules revealed typical unordered structure around apolar atoms.

Keywords ribonuclease T_1; mutation; hydrophobic interaction; free energy; simulation; molecular dynamics

Several experimental studies in recent years using protein engineering have been directed toward evaluating changes in the interactions of an enzyme with other molecules such as ligands and solvent molecules, and changes in the interactions within the enzyme molecule itself. Effects of mutations arise from the changes in interactions, involving hydrogen bonding, van der Waals, electrostatic and hydrophobic one. The accumulation of experimental results with systematic mutations has enabled us to elucidate some effects of mutation in a quantitative manner. Some of the results of protein engineering have been the subject of theoretical studies using a new approach called the free energy perturbation (FEP) method, which utilizes statistical mechanics and molecular dynamics (MD).[2] Based on the concept of the thermodynamic cycle, this technique has been applied to calculation of the difference in free energy changes of substrate binding or of activation for catalysis by the mutant and the wild-type enzyme.[3] These simulations have explained the changes in both energetic and structural aspects, and have allowed experimental results to be rationalized.

Ribonuclease T_1 (RNase T_1) is a small acidic protein secreted by *Aspergillus oryzae*. The gene of RNase T_1 was chemically synthesized and was expressed in *Escherichia coli* cells[4] for protein engineering studies. Based on the X-ray structure, a series of mutations were introduced at the guanine-binding site of the enzyme[4,5] as well as at the catalytic site,[6] and kinetic studies made on the hydrolysis of several substrates by the mutant enzymes. It was recently found that a mutant RNase T_1 in which Tyr45 is substituted by a more hydrophobic residue, tryptophan,[7] possesses a higher nucleolytic activity against pGpC than the wild-type enzyme with an increase in the initial velocity by 110—140%, in contrast to most mutant enzymes studied which exhibit lower activities than the wild-type enzyme. The kinetic parameters measured indicate that the enhancement of nucleolytic activity is due to a higher affinity of the mutant enzyme to the substrate than that of the wild-type enzyme. In this paper, we describe further analysis of the effects of the mutation (Tyr45→Trp) of RNase T_1 on the binding to its ligand using MD and FEP calculations.

Theoretical

The theory was developed by Kollman and coworkers,[2,3,8] and according to it, the free energy change ΔG on mutation from wild-type RNase T_1 to mutant RNase T_1 is given by[9]:

$$\Delta G = -RT \sum_{i=1}^{N} \ln \left\langle \exp\left(-\frac{H(\lambda_i) - H(\lambda_{i-1})}{RT} \right) \right\rangle_{\lambda_{i-1}} \quad (1)$$

where R is gas constant, T the absolute temperature, $\langle\ \rangle_\lambda$ the ensemble average at an intermediate position along the conversion pathway characterized by the coupling parameter λ, and $H(\lambda_i)$ the Hamiltonian for the states λ_i. A summation is taken over the N "thermodynamic windows." In our calculations, H was taken to be the potential energy function of classical molecular mechanics, and MD calculation was employed to obtain ensemble average of the molecular system. The Hamiltonian $H(\lambda)$ on the

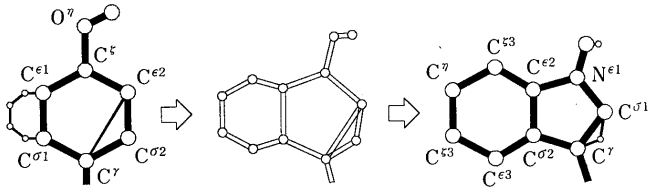

Fig. 1. A Schematic Diagram of the Computational Mutation from Tyrosine to Tryptophan

Dummy atoms, represented by small circles, were given zero values of electrostatic and van der Waals parameters. These atoms had no contribution to total potential energy values. Thick and thin lines represent actual and virtual bonds, respectively. In the intermediate state, the characteristics of chemical bonds are hybrids of those in pure tyrosine and in pure tryptophan.

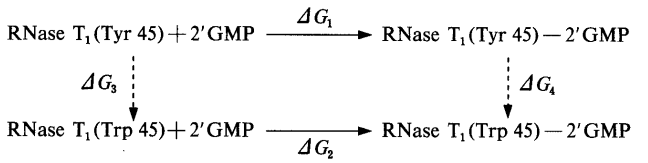

Fig. 2. Thermodynamic Cyclic Used in the Present Study

The ΔG_1 and ΔG_2 are obtained from gel filtration experiments. The ΔG_3 and ΔG_4 are calculated by MD/FEP method (broken line).

conversion pathway is defined by the parameter λ:

$$H(\lambda)=\lambda H_w+(1-\lambda)H_m \quad (2)$$

where subscripts w and m denote the wild-type and the mutant enzyme, respectively. For the smooth conversion from tyrosine to tryptophan or *vice versa*, we introduced virtual bonds between C^{δ} and C^{ε} atoms and between C^{γ} and C^{ε} atoms of Tyr45 ring as shown in Fig. 1. Then, we calculated free energy difference between the wild-type enzyme, RNase T_1 (Tyr45), and the mutant one, RNase T_1 (Trp45), both with 2′-guanylic acid (2′-GMP) (ΔG_4) and without 2′-GMP (ΔG_3). Corresponding chemical reaction never occurs in nature, however, the values obtained from computations could be easily compared with the experimentally obtained values. As shown in Fig. 2, because free energy is a state function and does not depend on the pathway between the states, the difference of binding free energy changes can be estimated as follows:

$$\Delta\Delta G=\Delta G_4-\Delta G_3=\Delta G_2-\Delta G_1 \quad (3)$$

where ΔG_1 and ΔG_2 are the binding free energy changes for RNase T_1 (Tyr45) and RNase T_1 (Trp45), respectively.

Method

Computational The initial structures of the RNase T_1–2′-GMP complex (*i.e.*, complex form) and the RNase T_1 alone (*i.e.*, free form) were taken from the crystal structures of RNase T_1 with 2′-GMP[10] and RNase T_1 with 3′-guanylic acid (3′-GMP),[11,12] respectively. The structure of RNase T_1 free form might resemble that of the complex with 3′-GMP rather than that of the complex with 2′-GMP due to looser binding of 3′-GMP to RNase T_1.

The crystal structures were first energy-minimized by the conjugate gradient method, until the energy gradient decreased to less than 2 kJ/mol/Å, applying energy constraints of 418 kJ/mol/Å^2 to all atoms. Then, a sphere of 17 Å radius centered on the C^{β} atom of Tyr45 was filled with TIP3P water molecules[13] simulated by the Monte Carlo method. Finally, the system "capped" with water molecules was energy-minimized again. The potential energy parameters were those of AMBER[14]/OPLS,[15] the dielectric constant was unity, and a non-bonded cutoff of 8Å was used.

The energy-minimized structures were subjected to MD calculations under constant temperature (310 K) and pressure. The energy parameters were the same as those in energy-minimization procedures. The SHAKE method[16] was used to fix bond lengths related to hydrogen atoms and a time step of 2 fs was used. Atoms within 17 Å of the C^{β} atom of Tyr45 were moved, and remaining atoms outside the sphere were fixed. After 80 ps equilibration, mutation was done in two stages with electrostatic decoupling. At the first stage, the atomic charges were changed while van der Waals parameters were left unchanged over 41 thermodynamic windows. Then, van der Waals radii and well depths were changed while electrostatic parameters were kept unchanged over 41 thermodynamic windows. Each window consisted of 400 steps of equilibration and 400 subsequent steps of data collection. In order to evaluate the precision of the calculations, forward conversion from RNase T_1 (Tyr45) to RNase T_1 (Trp45) and backward conversion in the reverse direction were done. To evaluate the structure of RNase T_1 (Trp45), structure were averaged over 20 ps in simulation for equilibration started from the end of the simulation for the forward conversion. Throughout this paper, the term "time-averaged structure" refers to this averaged structure of the mutant enzyme.

The calculations were done with program AMBER 3.0[14] on a VAX 8600/VMS computer at Taisho Pharmaceutical Co., Ltd. and an NEC SX/2 supercomputer at the NEC Corporation. Three dimensional structures obtained were inspected using programs QUANTA (Polygon Co., Ltd., MA, U.S.A.), MOLDISP II or PLUTO.[17]

Results and Discussion

Structure of RNase T_1 Energy-minimization applied to crystal structures until energy gradients were less than 2 kJ/mol/Å reduced the total potential energies from -12.0×10^3 kJ/mol to -14.6×10^3 kJ/mol for the complex form and from -8.6×10^3 kJ/mol to -13.4×10^3 kJ/mol for the free form. The root mean square (r.m.s.) deviations from crystal structures were 0.16 and 0.17 Å for the complex and the free forms, respectively. No significant changes in guanine-binding site were detected for either the hydrogen

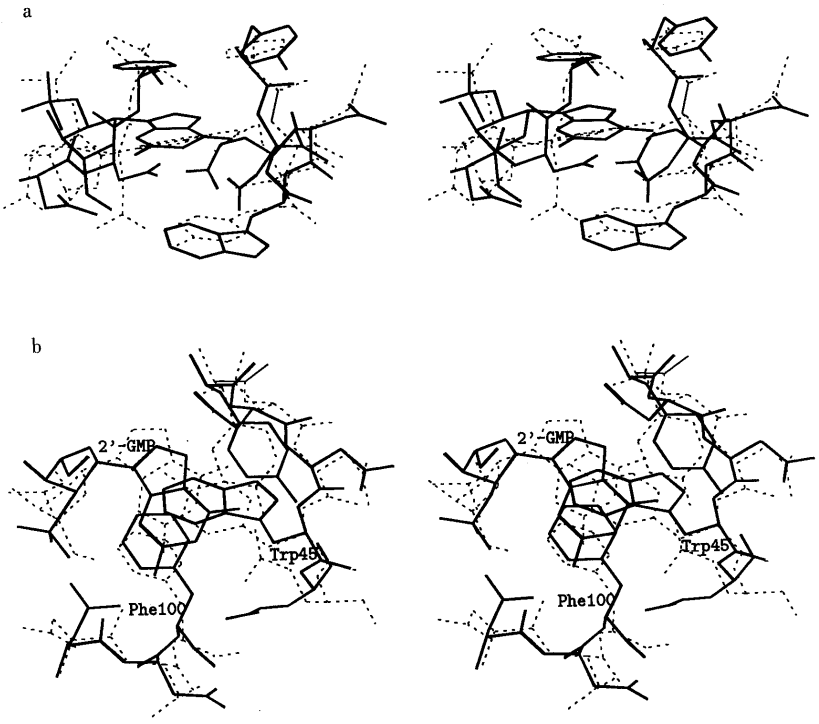

Fig. 3. Stereo-Views of the Guanine-Binding Site of RNase T_1–2′-GMP Complex

Time-averaged structure of the mutant RNase T_1–2′-GMP complex (solid bonds) is superimposed on the crystal structure of wild-type RNase T_1–2′-GMP complex (dotted bonds). a: view parallel to the guanine base plane, b: down to the plane.

TABLE I. Number of Atoms Considered in the MD Simulations

Atom	RNase T_1 (Free form)	RNase T_1–2′-GMP (Complex form)
Atoms moved	1618	1609
Atoms fixed	685	685
Atoms belonging to water	1347	1308
Total	2303	2294

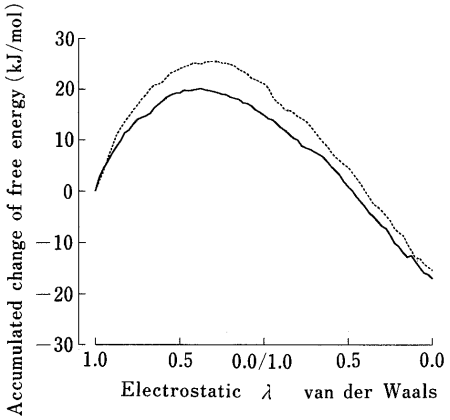

Fig. 4. Plots of Accumulation of Free Energy Changes

Simulation for the RNase T_1–2′-GMP complex (solid line) and that for the free enzyme (dotted line) are shown. Perturbations were done with electrostatic decoupling. Electrostatic perturbations are shown at left of center on the abscissa and van der Waals perturbations at right.

bonding or the stacking interactions. These results indicate that the starting coordinates were accurate enough for subsequent MD calculations.

As shown in Table I, total numbers of atoms considered were 2294 and 2303, for the complex and the free forms, respectively, the numbers of atoms fixed were 1609 and 1618, and the numbers of water molecules were 436 and 449. The smaller number of water molecules for the complex form was due to their expulsion by 2′-GMP molecule.

In Fig. 3, the time-averaged structure of guanine-binding site of RNase T_1 (Trp45)–2′-GMP complex is superimposed on the crystal structure of RNase T_1 (Tyr45)–2′-GMP complex. No significant changes occurred in the vicinity of the guanine-binding site of RNase T_1 after the mutation. Multidentated hydrogen bonds to the guanine base are found in both structures. Stacking of Trp45 on the guanine base is seen. The structural changes caused by conversion from RNase T_1 (Tyr45) to RNase T_1 (Trp45) were relatively small and localized; the mode of interaction between RNase T_1 and 2′-GMP was basically unchanged. There are, however, distinctions between these two structures and these are discussed later.

Calculated Difference of Binding Free Energy Changes between the Wild-Type and the Mutant (Trp 45) Enzymes Figure 4 shows the profiles of the accumulated free energy changes in the forward conversion process from Tyr45 to Trp45 with electrostatic decoupling; at the end of the conversion, the difference of binding free energy changes, $\Delta\Delta G$, is given as the difference between the free energy change for the free form (dotted line; ΔG_3 in Fig. 2) and the complex form (solid line; ΔG_4).

Table II lists respective values of calculated and experimental free energy changes. The agreement between

TABLE II. Changes in Free Energy (kJ/mol) Due to Conversion from RNase T_1(Tyr45) to RNase T_1(Trp45)[a]

Method	RNase T_1 ΔG_3	RNase T_1–2′-GMP ΔG_4	Difference $\Delta\Delta G$
Calculation	−15.1 (0.3)	−18.6 (1.6)	−3.4 (1.9)
Experimental	—	—	−2.5 (0.3)

a) The values calculated are averaged for forward and backward conversions, and halves of their absolute differences are given in parentheses. Values in the row Experimental are cited from T. Hakoshima *et al.*[18]

the two values for the difference of binding free energy changes is good, and the computational precision estimated from the differences between forward and backward conversions is within a few kJ/mol.

It should be noted that the mutation from Tyr45 to Trp45 gave a negative value for the difference of the binding free energy changes, indicating that the mutant enzyme has an energetic advantage over the wild-type one with respect to the binding to 2′-GMP. This could be due to the difference in hydrophobic properties of aromatic side chain of the 45th residue. When RNase T_1 is in a free form, both sides of this aromatic ring can be exposed to solvent. However, once 2′-GMP is bound to RNase T_1, this aromatic ring is stacked on the guanine base of 2′-GMP, and one side of the ring is no longer accessible to solvent molecules. In other words, accessible surface area for the aromatic ring of Tyr45 or Trp45 is reduced from 100% to 50% on binding to 2′-GMP. Thus, if the difference of binding abilities between the wild-type and the mutant enzymes is mainly due to the change in hydrophobic interactions, half of the difference between hydrophobicities of the side chains of tyrosine and tryptophan should correspond to the difference in the binding free energy changes between the mutant and the wild-type enzymes. Nozaki and Tanford reported the transfer free energy changes of amino acid side chain from water to ethanol or dioxan,[19] and the values for tyrosine and tryptophan were −9.6 kJ/mol and −14.2 kJ/mol, respectively. Thus, the replacement of tyrosine with tryptophan resulted in half (−2.3 kJ/mol) of the difference of these free energies. This value agrees well with the differences of binding free energy changes obtained experimentally and by our MD/FEP calculations. This could be a simple way of interpreting the mechanism for the increase of binding ability of the mutant enzyme to 2′-GMP.

Structural Difference between the Wild-Type and the Mutant Enzymes As mentioned above, the calculated value for the difference of binding free energy changes ($\Delta\Delta G$) agreed well with the experimental, allowing some evaluation of subtle structural changes caused by the substitution of Trp45 for Tyr45.

As shown in Fig. 3, the conversion increased the degree of stacking interactions not only between the guanine base and the aromatic ring of 45th residue, but also between this base and the aromatic ring of Phe100. This caused tighter binding of 2′-GMP to the mutant enzyme than to the wild-type enzyme.

Hydrogen bonds formed between the guanine base of 2′-GMP and the RNase T_1 play an important role in specific recognition of the base by RNase T_1. Table III is a list of the hydrogen bonds between RNase T_1 and guanine base

of 2′-GMP, both the crystal structure of the wild-type enzyme and for time-averaged structure of the mutant enzyme. There are some changes in hydrogen bonding interactions of the mutant enzyme with the guanine base compared to that of the wild-type enzyme. It is possible that this distinction is produced by the local conformational changes of the peptide backbone in the vicinity of the substituted 45th residue, and by shift of 2′-GMP binding position, rather than arising from local fluctuation of side chains. This possibility is strongly supported by the fact that the hydrogen bonding interactions found in the recently determined X-ray structure of the mutant enzyme (Tyr45) were almost identical to those obtained in our calculated structure of the mutant enzyme, as described later.

Distributions of Water Molecules The mutation of Tyr45 to Trp45 decreased binding free energy change. The reason the mutant enzyme with Trp45 binds to 2′-GMP more tightly than the wild-type enzyme can be understood simply as an increase in hydrophobic interactions, as stated earlier. In general, the change in hydrophobic interactions arises from change in the structure of surrounding water molecules. Table IV lists the number of water molecules within 5 Å from C^{β} atom of Tyr45 or Trp45. The radius of 5 Å seems sufficient since apolar solvation is characterized by solute-solvent distribution functions which peak between 3.5 and 3.9 Å from an apolar particle.[20] Number of water molecules for the free form is greater than that for the complex form, since in the former case water molecules occupy the space corresponding to an inhibitor molecule in the latter case. The number of water molecules was increased in the calculation order: equilibration period, electrostatic perturbation period and van der Waals perturbation period. It is noteworthy that the number of water molecules slightly

TABLE III. Hydrogen Bonds Between RNase T_1 and Guanine Base in 2′-GMP

Atom (RNase T_1)	Atom (2′-GMP)	Distance (Å)
In the wild-type enzyme		
N Asn43	O6	2.65
$N^{\delta 2}$ Asn43	N7	2.98
N Asn44	O6	2.64
$O^{\varepsilon 1}$ Glu46	N1	2.65
O Asn98	N2	2.75
In the mutant enzyme		
N Asn43	N7	2.79
N Asn44	O6	3.29
N Trp45	O6	2.59
$O^{\varepsilon 2}$ Glu46	N2	3.36
O Asn98	N2	3.04

TABLE IV. Distribution of Water Molecules Around Tyr45 or Trp45[a]

Period	RNase T_1		RNase T_1–2′-GMP	
	n	*r*	*n*	*r*
EQ[b]	79.9 (4.0)	7.8 (0.9)	65.9 (4.8)	6.2 (0.6)
EL[b]	80.9 (6.5)	8.1 (0.9)	68.2 (5.7)	7.3 (0.5)
VDW[b]	84.7 (7.2)	8.4 (0.7)	73.1 (5.9)	6.8 (0.4)

a) *n*: The number of atoms belonging to water within a 5 Å radius of the C^{β} atom, *r*: distance between center of the water sphere and the farthest atom of Tyr45/Trp45. *b*) Abbreviations EQ, EL and VDW denote equilibration, electrostatic perturbation and van der Waals perturbation, respectively.

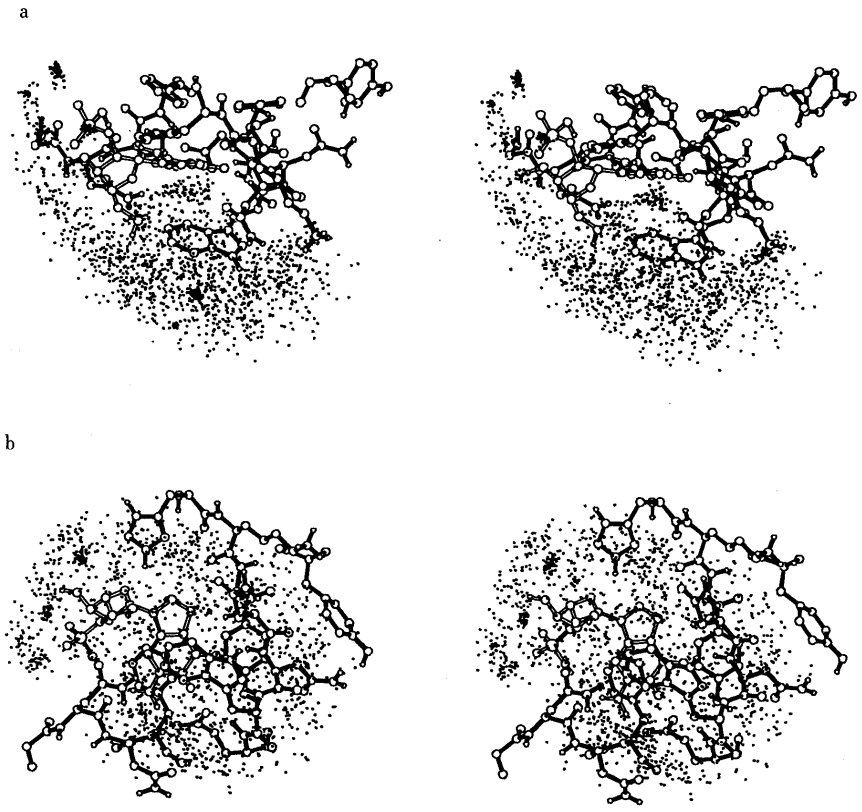

Fig. 5. Stereo-Views of the Distribution of Water Molecules in the Vicinity of the Guanine-Binding Site of the Mutant RNase T_1–2′-GMP Complex

Structure at the final stage of the perturbation is shown with water molecules (dots) within 5 Å from Trp45 indole ring sampled over 20 ps at each 4 ps interval. The bonds of part of the enzyme are solid and those of the 2′-GMP molecule are open. a: view parallel to the guanine base plane, b: down to the plane.

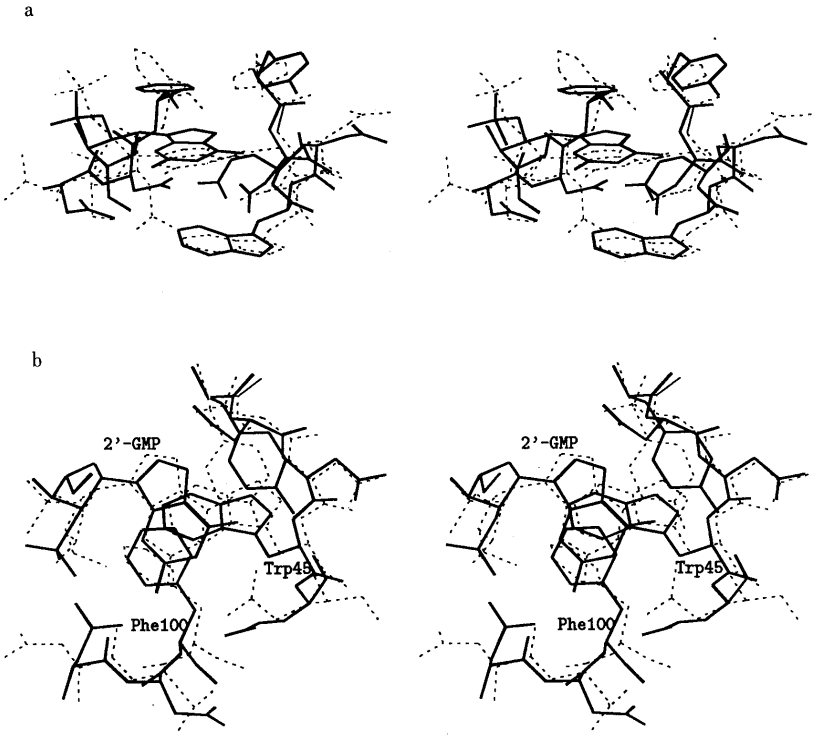

Fig. 6. Stereo-Views of the Guanine-Binding Site of the Mutant RNase T_1–2′-GMP Complex

Time averaged structure (solid bonds) is superimposed on the crystal structure of mutant RNase T_1–2′-GMP complex. a: view parallel to the guanine base plane, b: down to the plane.

increase as the conversion from tyrosine to tryptophan got closer to the final stage. On the other hand, the center of the distribution of water molecules revealed no significant shift judging from the distances presented in Table IV. The distribution of water molecules around the binding site at the last stage of conversion is shown in Fig. 5. No ordered structure formed by water molecules is detectable around the side chain of Trp45. There are no conflicting points on distribution of water molecules between our results and those of more detailed studies reported on solvation effects for protein dynamics.[20,21] In order to analyze the water structure in more detail, we will consider radial distribution functions, density, and diffusion constants.

Reliability of MD Simulation The crystal structure of mutant RNase T_1 (Trp45) complexed with 2′-GMP has recently been determined at 1.9 Å resolution by X-ray crystallography.[22] The difference between the crystal structure and the time-averaged structure calculated in this study is very small. The r.m.s. deviation of C^α atoms of the time-averaged structure from the crystal structure of the mutant enzyme was 1.10 Å. The guanine-binding sites of both structures are compared in Fig. 6 and the agreement between them is excellent, especially the stacking interactions between Trp45 and 2′-GMP which are almost identical. Furthermore, in the X-ray structure of mutant RNase T_1 (Trp45) complexed with 2′-GMP, atoms involved in hydrogen bonding interactions are almost identical to those presented in Table III for calculated structure of the mutant enzyme. The mean absolute difference of hydrogen bonding distances for both structures is only 0.37 Å. These results strongly support the reliability of our simulation study of mutation of RNase T_1.

This study was performed before and independently of the X-ray structural determination for mutant RNase T_1 (Trp45)–2′-GMP complex. If no X-ray structure of the mutant enzyme was available, detailed information on structural changes caused by mutation, as described here, could be obtained only by MD simulations on enzyme mutations. This is the greatest advantage of MD/FEP calculations as applied to mutations of enzymes.

To our knowledge, at least two MD calculations have been reported for RNase T_1. MacKerell *et al.*[23] reported MD simulations performed on free RNase T_1 using stochastic boundary conditions to understand the structure and fluctuations of the enzyme. They pointed out that the effect of water on the enzyme molecule was essential. In our calculations, hydration effects were also considered, and no undesirable structural changes were caused. Hirono and Kollman, on the other hand, reported MD/FEP calculations on the conversion of 2′-GMP complexed with RNase T_1 to 2′-adenylic acid resulted in good agreement with experimental value.[24] Thus, these studies as well as our results seem to support the validity of MD/FEP calculation for both energetic and structural analysis of protein-ligand interactions.

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References and Notes

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