Tautomeric stabilities of 4-fluorohistidine shed new light on mechanistic experiments with labeled ribonuclease A

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ABSTRACT. Ribonuclease A is the oldest model for studying enzymatic mechanisms, yet questions remain about proton transfer within the active site. Seminal work by Jackson *et al.* (Science, 1994) labeled Ribonuclease A with 4-fluorohistidine, concluding that active-site histidines act as general acids and bases. Calculations of 4-fluorohistidine indicate that the π -tautomer is predominant in all simulated environments (by ~17 kJ/mol), strongly suggesting that fluoro-labeled ribonuclease A functions with His119 in π -tautomer. The tautomeric form of His119 during proton transfer and tautomerism as a putative mechanistic step in wild-type RNase A remain open questions and should be considered in future mechanistic studies.

KEYWORDS. Fluorinated amino acids, histidine, RNA hydrolysis, enzymatic mechanism, tautomerization.

Introduction

Ribonuclease A (RNase A) is a 13.7 kDa enzyme that has long served as a model for understanding enzymatic mechanisms [1-3] and more recently, the role of dynamics in enzyme catalysis[4,5]. One of the seminal contributions to understanding proton transfer in RNA hydrolysis was made by Jackson *et al.* [6] In their groundbreaking report, 4-fluorohistidine (4-FHis) [7], which has a pK_a of ~ 3 [8], was incorporated at the His12 and His119 sites during the total chemical synthesis of RNase A. These two histidines participate in the two steps of RNA hydrolysis within the active site, as shown in Figure 1. In the labelling study with 4-fluorohistidine, it was found that catalysis was optimal at low pH values, consistent with the pK_a of 4-FHis, and supporting the notion that proton transfer in RNase A is not rate-limiting. However, some ambiguity in the histidines' roles, and the overall enzymatic mechanism, remain [9-11]. Recent analysis by Harris and co-workers declares, “effects of 4-fluorohistidine substitution on catalysis are only ca. 10-fold, suggesting a relatively small Brønsted effect, or that a nonchemical step is partially rate-limiting” [12].

Histidine in its neutral form exists as two tautomers (protonation states), as shown in Figure 2a. In aqueous solution, histidine exists in an 80:20 τ -tautomer: π -tautomer ratio. Of course, protein environments can shift the tautomeric equilibrium via altered dielectric environments and hydrogen bond donors and acceptors within the microenvironment around histidine residues[13-16]. We have recently published an investigation of the pH-dependent fluorine chemical shifts of 2- and 4-fluorohistidine, which are unusual. In that work, we showed that the upfield chemical shift that is experimentally observed during acid titration of 4-FHis [8] arises solely during

protonation of the π -tautomer, while protonation of the τ -tautomer results in a calculated downfield shift [17]. As we will show in our present work, this π -tautomer of 4-FHis is dominantly preferred.

Figure 1. Role of 4-fluoro-His119 and -His12 in the two proton transfer steps of RNA hydrolysis.

The strong thermodynamic preference for the π -tautomer of 4-fluorohistidine looks to have significant impact for interpretation of the results of 4-fluorohistidine labeled RNase A. The tautomeric states of histidine in RNase A were assigned by multiple researchers using NMR spectroscopy [18,19], neutron diffraction[20], and X-ray diffraction[21-29] (which of course does not definitively assign proton positions). Perhaps most relevant to understanding enzyme function is the tautomeric determination of the two RNase A active site histidines in aqueous solution (His12 and His119, shown

in Figure 1). Of these two residues replaced by 4-fluorohistidine in the work by Jackson *et al.*[6], His12 in solution state is identified to be 100% in the π -tautomer, via ^{13}C NMR and determination of micro- pK_a constants[18]. In contrast, His119 in solution has been identified to exist in a τ : π tautomeric ratio of 85:15 [18,19], which is close to the innate tautomeric stability of histidine in water (80:20)[30]. Further complicating analysis is the conformational flexibility of His119:[31] it has been shown to have pH-dependent positions[32,33].

Figure 2. Tautomeric stabilities for a) histidine [30] and b) 4-fluorohistidine.

Computational/Experimental methods

Optimized geometries were obtained using Gaussian 09[34] software at different levels of theory (MP2/6-31++G(d)[35] and implicit CPCM water solvation [36] for

results in main text). Other methods (DFT and HF with different basis sets; SMD solvation model [37] in various solvents) were used to ensure that tautomeric stabilities are consistent across electronic structure methods. In order to probe the tautomeric stabilities of 4-fluorohistidine in different simulated peptide environments, tautomeric stabilities of 4-fluorohistidine analogues (N-acetyl-O-methyl-4-fluorohistidine or “capped 4-FHis”; zwitterionic 4-fluorohistidine with an intramolecular hydrogen bond; 4-fluoro-5-methyl-imidazole), and NAc-4-FHis-OMe in various dielectric environments were calculated. These are presented in Tables 1 and 2, respectively. Frequency calculations were performed to obtain the free energies at 310 K with the same electronic structure methods. Initial geometries were prepared using Gauss View 5 software[38]. ^{13}C NMR chemical shifts with the GIAO method[39] were calculated at the MP2/6-31++G(d) level, with CPCM water solvation, from optimized geometries with the same method. A scaling factor of 1.08 was used to give the reported ^{13}C chemical shifts[40,41].

^{13}C NMR experiments were conducted on a Varian Mercury VX 300 MHz NMR equipped with a Varian 300 SW/BB probe. 50 mM samples of 4-fluorohistidine were prepared in 500 μL of DI H_2O . A standard proton-decoupled ^{13}C NMR pulse sequence was used for acquisition, with a D_2O coaxial insert to provide a locking signal in order to acquire a 4-fluorohistidine only spectrum. The spectrum was then reacquired using a coaxial insert containing $\text{d}_6\text{-DMSO}/1\%$ TMS for internal reference, and the original spectrum was shifted so that both carbonyl peak ppm values match.

Discussion and Results

The tautomeric stabilities of 4-fluorohistidine, using multiple electronic structure methods, are given in Table 1. Different analogues of 4-fluorohistidine were evaluated,

in order to determine whether structural factors, such as intramolecular hydrogen bonds (provided in zwitterionic 4-fluorohistidine) and backbone effects (4-fluoro-(5-methyl)-imidazole versus NAc-4-FHis-OMe or “capped 4-FHis”) perturb the tautomeric equilibrium. It can be seen that, indeed, structural effects shift the relative stabilities, from ~17 kJ/mol for capped 4-FHis to ~23 kJ/mol for its methyl-imidazole analogue. Meanwhile, intramolecular hydrogen bonds stabilize the τ -tautomer somewhat, reducing its relative energy to ~15 kJ/mol higher than the π -tautomer. The effects of different dielectric environments on the tautomeric equilibrium of 4-fluorohistidine were also investigated, since dielectric variation within protein environments affects the tautomeric stabilities of histidine. These values are presented in Table 2, using MP2/6-311++g(d) energies in two different implicit solvation models, SMD and CPCM. It can be seen that regardless of dielectric environment, the π -tautomer is strongly favored, by over 17 kJ/mol.

Table 1. Tautomeric stabilities of 4-fluorohistidine and analogues at different levels of theory. All use aqueous, CPCM implicit solvation. The free energy values at 310 K are energies of the τ -tautomer relative to the π -tautomer (which is set to zero).

Method	“capped”4-FHis (NAc-4-FHis-OMe) without intramolecular hydrogen bond ΔE ($\tau - \pi$ tautomer)	4-FHis zwitterion with intramolecular hydrogen bond to protonated imidazole nitrogen ΔE ($\tau - \pi$ tautomer)	4-fluoro-(5-methyl)-imidazole no hydrogen bond; different inductive effects at C _{α} ΔE ($\tau - \pi$ tautomer)
MP2/6-31++g(d)	17.07 kJ/mol	15.23 kJ/mol	23.97 kJ/mol
B3LYP/6-31++g(d)	17.86 kJ/mol	15.77 kJ/mol	23.13 kJ/mol
BHandHLYP/6-31+g(d)	20.00 kJ/mol	16.11 kJ/mol	23.64 kJ/mol
M062x/6-31+g(d)	16.77 kJ/mol	17.53 kJ/mol	21.34 kJ/mol
HF/6-311++g(3df,2p)	17.23 kJ/mol	12.38 kJ/mol	22.17 kJ/mol
B3LYP/6-31+g(d)	20.33 kJ/mol	14.89 kJ/mol	23.13 kJ/mol
Average	18.2 kJ/mol	15.3 kJ/mol	22.9 kJ/mol
Standard deviation	1.6 kJ/mol	1.7 kJ/mol	1.0 kJ/mol

Table 2. Relative free energies at 310 K for “capped” 4-FHis (N-acetyl-O-methyl-4-fluorohistidine) τ -tautomer compared to stable π -tautomer in different dielectric environments with CPCM and SMD solvation models.

Method	Water ($\epsilon=78$) ΔE ($\tau - \pi$ tautomer)	Acetonitrile ($\epsilon=35$) ΔE ($\tau - \pi$ tautomer)	Ethanol ($\epsilon=25$) ΔE ($\tau - \pi$ tautomer)	Tetrahydrofuran ($\epsilon=7$) ΔE ($\tau - \pi$ tautomer)
MP2/6-31++g(d) CPCM solvent	17.07 kJ/mol	18.49 kJ/mol	18.41 kJ/mol	17.78 kJ/mol
MP2/6-31++g(d) SMD solvent	24.02 kJ/mol	20.54 kJ/mol	21.09 kJ/mol	17.53 kJ/mol

It is remarkable how strong the thermodynamic preference is for the π -tautomer of 4-fluorohistidine. This is a much greater difference in tautomeric stabilities than what is seen in the native amino acid histidine. With histidine’s intrinsic τ -tautomeric stability (favored by ~ 4 kJ/mol), there is an 80:20 τ : π equilibrium in aqueous solution. For 4-fluorohistidine’s π -tautomeric stability, the π -tautomer population is 99.9% at 310 K. This strong preference for the π -tautomer (by ~ 17 kJ/mol, or $6.5k_B T$ at 310 K) remains regardless of electronic structure methods or simulated changes in protein environment. The strong preference for the π -tautomer appears to be due to the inductive effect of fluorine next to N ϵ 1 (protonated in the τ -tautomer), which drastically drops its pK_a value relative to N δ 2 (protonated in the π -tautomer). We have also acquired the ^{13}C NMR spectrum for 4-fluorohistidine, and assigned the resonances using electronic structure methods. This is shown in Figure 3, where calculated imidazole ring ^{13}C shifts for the π -tautomer (solid blue bars) and τ -tautomer (dashed red bars) are plotted for comparison. The π -tautomer appears to be dominant in the ^{13}C NMR spectrum.

In the classic RNase A catalytic mechanism (Figure 1), it is widely accepted that His12 is in the π -tautomeric form. This may be one reason why synthetic incorporation of 4-fluorohistidine into RNase A was feasible and yielded an active enzyme: the fluorinated histidine analogue maintains the tautomeric preference of the enzyme. Meanwhile, the tautomeric form of His119 during proton transfer steps has not been definitively assigned, but recently developed methods for measuring histidine tautomers in transient protein conformations may be able to address this question[14].

His119 is often illustrated in the π -tautomeric form [3] [6] [10] [12], while recent modeling appears to have His119 in τ -form [10] [12]. Work by Schultz *et al.* suggests that the major function of Asp121 is to properly orient the correct tautomer of His119, in which the τ -tautomer (N δ 1 deprotonated, N ϵ 2 protonated) is favored[22]. However, λ -dynamics simulations by Khandogin and Brooks suggest a thermal equilibrium of both tautomers [42]. It is known from the 4-fluorohistidine labeling experiment that His119 acts as a general acid, and, interestingly, other work has shown that His119 can exist in two forms (A and B) that may depend on the catalytic step.[18] [19]

Intriguingly, the two active site histidines in microbial RNases have been shown to be in the π -tautomer, even with energetic penalties. The two π -tautomeric active-site histidines are a conserved feature in microbial ribonucleases and ribotoxins. Histidine tautomeric state is related to microbial ribonuclease function, with Pérez-Cañadillas *et al.* noting its influence on pK_a values of neighboring residues and transition state stabilization through electrostatic and structural interactions[33].

Figure 3. ^{13}C NMR spectrum of 4-fluorohistidine in H_2O (dark red). Calculated chemical shifts for π -tautomer (blue bars) and τ -tautomer (red dashed bars) are plotted for comparison.

Conclusions

The ramifications of the strong favorability for the π -tautomer of 4-fluorohistidine are two-fold in the ribonuclease A experiment. First, it can be seen from the results of Jackson *et al.* that catalysis by fluoro-labeled RNase A is efficient when both His12 and His119 are in the π -tautomeric form[6]. Secondly, it raises the question whether tautomerization contributes to the overall catalytic rate in wild-type RNase A, since the His119 tautomeric ratio is 85:15 $\tau : \pi$, yet proton transfer presumably takes place via the π -tautomer when RNase A is labeled with 4-fluorohistidine. If histidine tautomerism is a mechanistic step in ribonuclease catalysis, then labeling with 4-fluorohistidine obviates tautomerization. Which tautomer of His119 participates in the native enzyme during catalysis appears to be an open question, and should be considered when designing and

interpreting future simulations and experiments. With these present results, the seminal work by Wells and co-workers can be re-evaluated for even more, valuable mechanistic information.

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ribonuclease A labeled with 4-fluorohistidine

π -tautomer
strongly favored

wild-type ribonuclease A

tautomeric form
unknown