Tautomeric state of α-sarcin histidines. Nδ tautomers are a common feature in the active site of extracellular microbial ribonucleases

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Abstract Extracellular fungal RNases, including ribotoxins such as α-sarcin, constitute a family of structurally related proteins represented by RNase T1. The tautomeric preferences of the α-sarcin imidazole side chains have been determined by nuclear magnetic resonance and electrostatic calculations. Histidine residues at the active site, H50 and H137, adopt the Nδ tautomer, which is less common in short peptides, as has been found for RNase T1. Comparison with tautomers predicted from crystal structures of other ribonucleases suggests that two active site histidine residues with the Nδ tautomer are a conserved feature of microbial ribonucleases and that this is related to their ribonucleolytic function.

Key words: Histidine tautomer; pK_a; α-Sarcin; Ribonuclease; Ribotoxin

1. Introduction

Ribotoxins are highly specific ribonucleases which share a high degree of sequence identity [1], almost identical structures [2,3] and modes of action [1,4]. α-Sarcin, produced by the mold *Aspergillus giganteus* [5,6] is the best characterized member of this family of fungal cytotoxins [7,8], and its solution structure [2], electrostatics [9] and dynamics [10] have been extensively studied. This secreted ribotoxin inhibits protein biosynthesis in target cells by cleaving a single phosphodiester bond in a strictly conserved RNA sequence [11–13] located in the 'sarcin/ricin' loop (SRL) of the large ribosome subunit [14,15]. It is a small (150 residue) and highly basic protein (p*I* 9.4) containing four Arg, eight His and 20 Lys residues. The histidine residues are widely distributed throughout the protein structure; some are in regular secondary structure elements: α-helix (His 35 and His 36), central β-sheet (His 50 and His 137); others in loops: loop 2 (His 82 and His 92), loop 3 (His 104); and the C-terminus (His 150) (Fig. 1). His 50, His 82, His 137 and His 150 have altered pK_a values relative to model compounds and their participation in electrostatic and structural interactions has been reported [9]. A salt bridge involving His 82 links loops 1 and 2 and has been proposed to contribute to the conformational stability. His 150 interacts with the N-terminal region, assisting a disulfide bridge (Cys 6-Cys 148) maintain the correct orientation of the N-terminal β-hairpin (important for the SRL interaction) with the central β-sheet. Moreover, histidines 50 and 137 are at the active site (Fig. 2) and their roles in catalysis have been determined [9,16,17].

The structural and electrostatic properties of histidine residues depend on the ionization and tautomeric equilibria of their side chains. The imidazolium cation produces two different imidazole tautomers upon proton dissociation at high pH, a free Nδ or a free Nε site. Whereas the tautomeric state is not determined by X-ray crystal or nuclear magnetic resonance (NMR) structures, it can be derived from heteronuclear chemical shifts by 2D NMR methods [18,19]. ^{13}C NMR studies on free histidine have shown a four-to-one preference for the Nε–H tautomer [18]. Moreover, studies on Nε or Nδ methylated histidine derivatives reveal that the Nδ microscopic pK_a is 0.6 pH units lower than for Nε [18,20]. The association of specific tautomeric states of histidine with metal ligation [21], catalysis [22] and phosphorylation [23] illustrates the importance of His tautomerization to biological function.

To better understand the roles played by α-sarcin histidines in both stabilizing the tertiary structure and in catalysis, we have analyzed the tautomeric states of the imidazole side chains of α-sarcin by the well-established ^{15}N–^{1}H 2D NMR

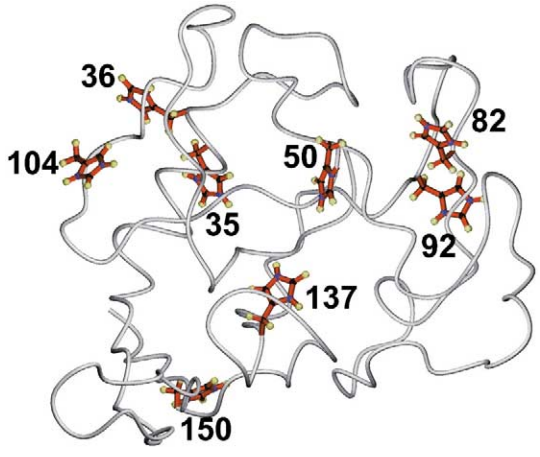

Fig. 1. Ribbon representation of the solution structure of α-sarcin illustrating the location of the histidine residues.

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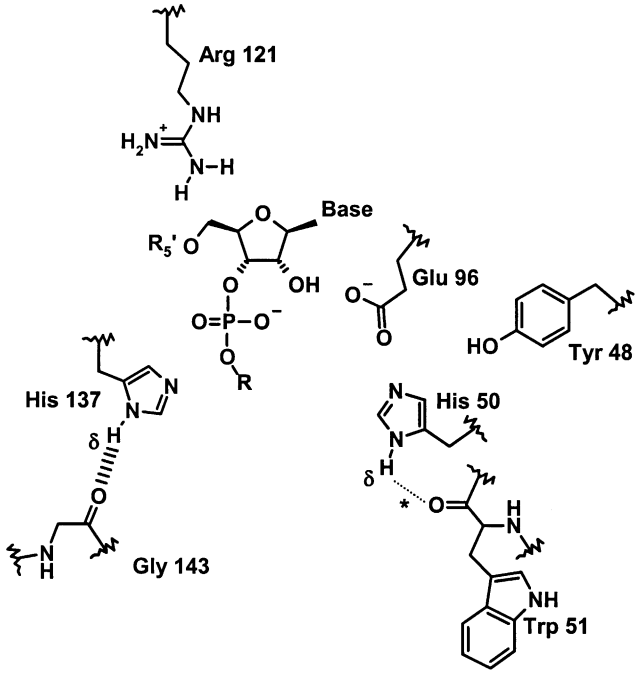

Fig. 2. Schematic drawing of the α-sarcin active site. Side chains of the catalytic (Glu 96 and His 137) and nearby residues (Tyr 48, His 50, Trp 51 and Arg 121) are shown. His 50 and His 137 are represented in the neutral state in their preferred tautomer found in the protein. *Hydrogen bond found in the homologous restrictocin structure.

methodology [19,24] and electrostatic calculations [25,26]. The results reveal that the tautomeric states of the histidines are important for α-sarcin's stability and catalytic function.

2. Materials and methods

2.1. NMR

Samples were prepared by dissolving the ^{15}N-labeled wild-type protein as described previously [9,27]. All spectra were acquired on a Bruker AMX-600 spectrometer. Two-dimensional ^{1}H–^{15}N HMQC spectra [28] of α-sarcin were acquired in D_2O at pH 8.5 and 35°C. The delay during which ^{15}N and ^{1}H signals become antiphase was set to 22 ms to refocus magnetization arising from $^{1}J_{NH}$ couplings. A total of 80 scans were signal-averaged for each of the 128 complex t_1 points. The ^{15}N carrier was set to 210 ppm. Spectra were processed with Bruker software.

2.2. Theoretical pK_a calculations

Calculations were performed on the family of 20 NMR α-sarcin structures (1DE3) to obtain averaged pK_a values for titratable residues by applying a continuous electrostatic approach that relies on the finite difference solution of the Poisson–Boltzmann equation (FDPB) as implemented in the University of Houston Brownian Dynamics software package (UHBD) [29,30]. Protocols from Wade and co-workers [25,26], which explicitly account for multiple site titration, were used. The dielectric constant was set to 78.5 for solvent and to 15 or 78.5 for sites in the protein depending on their calculated desolvation energy [25]. Calculations were performed at 35°C with the ionic strength set to 200 mM. Model compound pK_as from Nozaki and Tanford [31] were used for the reference state.

3. Results

3.1. Experimental determination of the tautomeric state of the α-sarcin histidine residues

The tautomeric states of α-sarcin histidines were derived from the pattern of cross-peaks and ^{15}N chemical shifts of the Nε and Nδ atoms observed in the long-range ^{1}H–^{15}N correlation spectrum shown in Fig. 3. In such spectra, cross-peaks of approximately equal intensity are observed for the Nε–Hε, Nε–Hδ and Nδ–Hε two-bond correlations [32], while the cross-peak for the Nδ–Hδ three-bond correlation is weak or absent [32]. Neutral histidines are identified by well-separated nitrogen resonances, 167.5 ppm for protonated nitrogen and 249.5 ppm for unprotonated nitrogen [19]. In charged histidine, both nitrogens resonate around 176.5, with the Nδ atom generally appearing about 1 ppm lower than the Nε atom [19]. In the α-sarcin spectrum at pH 8.5, signals corresponding to His 35, 50, 92, 137 and 150 are clearly observed and the tautomer of the neutral state was determined on the basis of the cross-peak pattern (Table 1). The Nδ nuclei of His 35, 50, 92, 137, and 150 resonate at 240, 178, 228, 166, and 186 ppm, respectively, and the chemical shifts of the corresponding Nε are 171, 203, 184, 248, and 206 ppm, respectively (Fig. 3). Assuming that these tautomeric equilibria are on the NMR fast-exchange regime, ^{15}N chemical shifts can be used semiquantitatively to estimate populations. Using this approach, the neutral state tautomers of His 137 and His 35 were found to be Nδ–H and Nε–H ($\geq 90\%$), respectively. Both these histidines are observed to be completely neutral at pH 8.5; this is consistent with their low macroscopic pK_as [9]. His 92 is also mainly neutral and the reduced difference between ^{15}N chemical shifts can be explained by a fast equilibrium (80:20) favoring the Nε–H tautomer. His 50 and His 150 with high pK_as are incompletely titrated and partially charged (56% and 39%, respectively) at pH 8.5. Strikingly, the intrinsically less common Nδ tautomer is predominant for these side chains; neutral His 50 is 84% Nδ and neutral His 150 is 70% Nδ.

Ambiguous patterns were found for His 36 and His 104 and no cross-peaks were detected for His 82, probably due to line broadening. For His 36, the observation of a broad signal centered at 206 ppm assignable to either or both ring nitro-

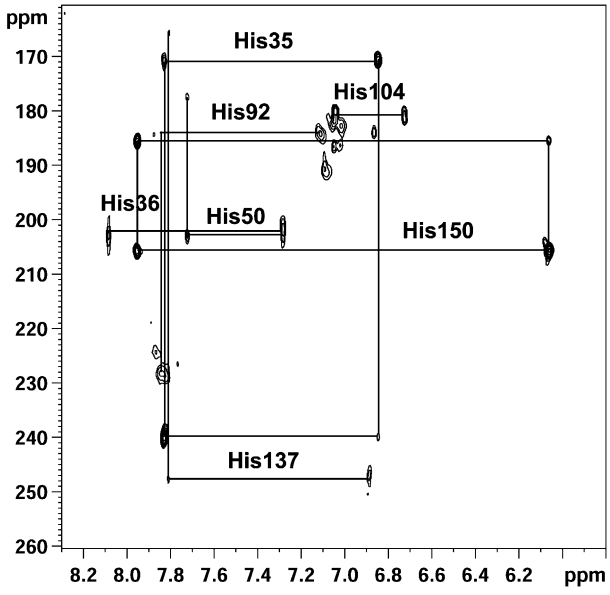

Fig. 3. ^{1}H–^{15}N HMBC spectrum of ^{15}N-labeled α-sarcin. Spectra were taken at pH 8.5 and 308 K.

Table 1
Experimental and calculated pK_a and tautomeric forms of histidine residues of α-sarcin

Residue	Experimental		Calculated pK_a		ΔpK_a[c]
	tautomer[a]	pK_a[b]	tautomer Nε	tautomer Nδ	
His 35	Nε	6.3 ± 0.2	6.5 ± 0.2[d]	–	−0.2
His 36	Nδ/Nε	6.8 ± 0.2	6.9 ± 0.1[e]	7.1 ± 0.1[e]	−0.1/−0.3
His 50	Nδ	7.7 ± 0.2	–	7.2 ± 0.1[e]	0.5
His 82	Nε	7.3 ± 0.1	7.8 ± 0.1[e]	–	−0.5
His 92	Nε	6.9 ± 0.1	6.6 ± 0.1[e]	–	0.3
His 104	Nδ/Nε	6.5 ± 0.2	5.4 ± 0.2[e]	5.9 ± 0.2[e]	1.1/0.6
His 137	Nδ	5.8 ± 0.1	–	5.6 ± 0.2[d]	0.2
His 150	Nδ	7.6 ± 0.1	–	6.9 ± 0.1[e]	0.7

[a]Tautomer assignment for each histidine was made: on the basis of NMR data, His 35, His 36, His 50, His 92, His 137 and His 150; on the basis of the 3D structure, His 82; and on the basis of the electrostatic calculations, His 104.
[b]Macroscopic experimental pK_a [9].
[c]ΔpK_a = pK_a(experimental)−pK_a(calculated).
[d]'Low dielectric' protein site (dielectric constant, 15).
[e]'High dielectric' protein site (dielectric constant, 78.5).

gens and quite close to the value estimated for a 1:1 Nδ:Nε tautomeric equilibrium (208.5 ppm) is evidence that this residue exists as a mixture of tautomers. As only one nitrogen signal centered at 181 ppm could be assigned to His 104, its tautomeric state could not be determined on the basis of NMR data alone.

3.2. Calculation of His pK_a values and tautomeric states

Since averaged computed pK_as have been reported to be more accurate than those based on a single structure [30], we performed calculations using the family of α-sarcin NMR structures and different tautomeric states for the neutral forms of histidine residues. The results summarized in Table 1 correspond to calculations performed with the tautomers determined by 2D-NMR (see above), except for His 82 and His 104 (see below). The closeness of the Nδ and Nε calculated pK_as for His 36 corroborates the 1:1 tautomeric ratio determined by their ^{15}N resonances. In general, experimental and calculated values are in good agreement.

While no ^{1}H–^{15}N cross-peaks were observed for His 82, the 3D NMR structure shows a hydrogen bond between its Nε proton and the Asp 41 side chain [2]. This is evidence that the Nε tautomer predominates, and this line of reasoning is supported by the close agreement of the experimental and calculated pK_a values. Alternative calculations considering different dielectric environments for the tautomers of His 104 show that although this site is identified as having a low dielectric constant by the UHBD program (values in Table 1), using the high value for the dielectric constant reproduces the experimental pK_a values better. Dynamic processes in this region revealed by ^{15}N relaxation studies [10] likely affect the dielectric properties around this residue. The fact that the current version of UHBD does not consider dynamic processes probably explains its inappropriate choice of the dielectric constant at this site.

Regarding the active site (Fig. 2), important pK_a differences are observed when calculations with different tautomers of His 50 and 137 were performed (Table 2). The pK_a of Glu 96 clearly depends on the tautomeric state of His 50 but not on that of His 137. Interestingly, the Arg 121 pK_a is strongly altered, up to 1.3 pH units, depending on the tautomeric states of His 137 and His 50. The experimental pK_as of active site residues are better reproduced when the His 137 Nδ and His 50 Nδ combination is chosen.

4. Discussion

Determination of the tautomeric preferences of the α-sarcin histidines is of special interest to better describe its active site as well as those of other members of this conserved family of extracellular ribonucleases. It is well-established that in α-sarcin Glu 96 and His 137 serve as the general base and acid, respectively, in catalysis, whereas the other histidine, His 50, is likely involved in transition state stabilization during the transesterification reaction [16]. The catalytic activity of α-sarcin depends on the delicate balance of structural and electrostatic interactions, which are affected by the tautomeric state of the histidines. In particular, different hydrogen-bonding networks can be established by different tautomers. The tautomeric state of α-sarcin His 137 determined in this work is that expected from the 3D structure in solution since its Nδ–H is hydrogen-bonded to the oxygen of Gly 143. The equivalent proton also is hydrogen-bonded in the α-sarcin homolog restrictocin [3] and in other microbial RNases whose

Table 2
Dependence of the averaged calculated pK_a values of the α-sarcin active-site residues as a function of the histidine tautomeric states

	Experimental[a]	Calculated pK_a		
		50Nε/137Nε	50Nδ/137Nε	50Nδ/137Nδ
Tyr 48	–	9.8 ± 0.1	9.7 ± 0.1	9.8 ± 0.1
His 50	7.7 ± 0.2	6.1 ± 0.1	7.1 ± 0.1	7.2 ± 0.1
Glu 96	5.2 ± 0.1	3.9 ± 0.1	4.7 ± 0.1	4.7 ± 0.1
Arg 121	–	12.7 ± 0.1	13.3 ± 0.1	14.0 ± 0.1
His 137	5.8 ± 0.1	4.9 ± 0.1	5.0 ± 0.1	5.6 ± 0.2

[a]Macroscopic experimental pK_a [9].

substrate-free structure is known [33,34]. This H-bond affects the electrostatics and structure of the protein [2]. In contrast, the NMR structure clearly shows that the His 50 side chain is not involved in hydrogen bonds, so the Nε tautomer which is more stable in solution [32] is expected to be present. The NMR data and the electrostatic calculations show, however, that it exists predominantly in the less common Nδ form. This is also the tautomer deduced from the crystal structure of the homologous ribotoxin restrictocin [3] whose corresponding His 49 Nδ–H is hydrogen-bonded.

Differences in ΔG can be calculated from shifts in the tautomer populations. Maintaining a histidine residue in the disfavored Nδ tautomer is not energetically free but destabilizes the folded conformation of α-sarcin about 2.2 kcal/mol per His or 4.4 kcal/mol for both active site residues. Why is the protein willing to bear such a substantial decrease in stability? The answer, we believe, is revealed by the experimental pK_as and those calculated for the various combinations of His active-site tautomers (Table 2). Namely, altering these tautomers would change the pK_as of the active-site groups and decrease the activity of the protein by reducing or acid-shifting the activity profile.

Ribotoxins also belong to the larger family of fungal extracellular ribonucleases, usually represented by RNase T1 [35,36]. These RNases have different biological functions and enzymatic activities, but all them share a common structural fold [2,3]. These fungal ribonucleases (St, T1, F1, Ms, U1, and U2) have two histidines at the active site with the same conformation as His 50 and His 137 in α-sarcin [1,36]. Recently, the histidine tautomers of RNase T1 have been reported [37] and show that both active site residues, His 40 and His 92, also adopt the less common Nδ tautomer in solution though none of these histidine rings is hydrogen-bonded in the T1 NMR structure [38]. In spite of the known structural differences between α-sarcin and RNase T1, these results highlight the similarity of their active sites. No other study on histidine tautomeric states has been reported for other members of this family. However, an analysis of the hydrogen-bonding pattern of active-site histidines can give some insight into the more probable tautomeric forms. Based on the known 3D structures, His 40 and His 92 in RNase F1 (1FUS) [39], His 41 and His 101 in RNase U2 (1RTU) [34], His 39 and His 91 in RNase Ms (1RMS) [40], have the Nδ histidine proton involved in hydrogen bonds. In all cases, the less common Nδ tautomer is predicted, suggesting a conserved structure–function link with their ribonucleolytic activity. With lower levels of sequence similarity, bacterial ribonucleases, like barnase and RNase Sa, are more distant cousins of the fungal ribonucleases, yet they also conserve an active-site His homologous to His 137 in α-sarcin. This His has just been shown to adopt the Hδ tautomer in RNase Sa [41].

This agrees with the hypothesis that microbial ribotoxins are naturally engineered proteins derived from ribonucleases with additional domains extended from the conserved catalytic core [42].

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