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Ribonuclease A mutant $His^{119}Asn$: the role of histidine in catalysis

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Abstract Bovine pancreatic ribonuclease A (RNase A) has been widely used as a convenient model for structural and functional studies. The enzyme catalyzes cleavage of phosphodiester bonds in RNA and related substrates. Three amino acid residues located at the active site of RNase A (His^{12}, His^{119}, and Lys^{41}) are known to be involved in catalysis. Mutation of His^{119} to asparagine was generated to study the role of His^{119} in RNase A catalysis. The mutant enzyme has been isolated and characterized. The mutation significantly decreases the rate of the transesterification reaction and has no effect on substrate affinity of the enzyme. An analysis of the enzymatic properties of H119N RNase A suggests that the imidazole ring of His^{119} of the wild-type enzyme must be protonated in an enzyme-substrate productive complex. Thus our results indicate that a contribution of protonated His^{119} into the catalysis is not restricted to protonation of oxygen atom of the substrate leaving group and that His^{119} participates directly in a transition state stabilization via hydrogen bonding.

Key words: Ribonuclease; RNase A; Site-directed mutagenesis

1. Introduction

Bovine pancreatic ribonuclease A (RNase A) and related proteins of the ribonuclease superfamily catalyze phosphodiester bond cleavage after 3′-pyrimidine bases in RNA and related substrates [1]. The RNase A catalyzed transphosphorylation reaction results in formation of RNA fragments terminated at the 3′-end with a 2′,3′-cyclic phosphodiester group [2]. In addition, the enzyme can accelerate hydrolysis of pyrimidinenucleoside-2′,3′-cyclophosphates into nucleoside-3′-phosphates. The tertiary structure of RNase A and its complexes with substrate-like ligands have been solved by X-ray crystallography and NMR methods [2–6]. Three catalytic residues at the active site of RNase A (His^{12}, His^{119}, and Lys^{41}) are believed to form hydrogen bonds with phosphate and ribose moieties of the RNA substrate in the transition state (Fig. 1). It was also shown that in native RNase A the imidazole ring of His^{119} may adopt two different conformations: A or B [4,6]. The side chain of His^{119} was found to be fixed in the "A" conformation in complexes of the enzyme with non-hydrolyzed dinucleosidemonophosphate analogs [4,7–9] and in the "B" conformation in complexes with the 3′-CMP-product of hydrolysis of the corresponding cyclophosphate [4,7]. A stabilization of the "A" conformation results from a stacking interaction of the downstream adenine of analog with His^{119} imidazole ring. Structural data indicate that the His^{119} must be in the "A" conformation to be able to form hydrogen bonds with a substrate in transition state in the course of the transesterification reaction [4].

According to the widely accepted "in-line" mechanism, His^{12} acts as general base by accepting a proton from the 2′-OH group of ribose while His^{119} acts as general acid by donating a proton to a leaving group in the transition state of the transesterification reaction. The role of these histidines is reversed during hydrolysis of cyclophosphates [10–12]. The following questions concerning participation of His^{119} in catalysis remain to be answered:

1. Is the catalytic function of His^{119} confined to a role as general acid in the phosphodiester cleavage reaction?
2. Does the ground state stacking interaction between the side chain of His^{119} and the adenine ring of the substrate UpA contribute to the enhanced rate of UpA cleavage of RNase A?

Recently, His^{119} was mutated to alanine and steady-state kinetic parameters for this RNase A mutant were estimated [13]. This mutation led to a decrease of k_{cat}/K_M values by 10^4-fold during cleavage of poly(C) and by almost 10^4-fold during cleavage of UpA substrates. These results show an important contribution made by His^{119} into RNase A catalysis but the precise role of this active site residue cannot be deduced from these data. The side chain of alanine is neither able to stabilize the transition state of the cleavage reaction by any mechanism nor to restrict conformational freedom of the phosphodiester bond to be cleaved. In addition, the alanine substitution at position 119 could disturb the hydrogen bond network and/or the water structure present in the active site of the enzyme. This paper describes the generation and purification of a H119N mutant RNase A and the effects of the asparagine substitution at position 119 on RNase A catalysis is determined.

2. Materials and methods

Chemically pure reagents were obtained from Merck and Sigma. Mobile phases were prepared with HPLC-grade acetonitrile (Fluka AG) as a strong solvent. The aqueous phases were prepared using Milli-Q water (Millipore).

All components for growth media were from Difco Laboratories (Detroit, MI). *Escherichia coli* strains JM109 and XL-1 Blue MRF' (Stratagene) were used for DNA manipulations and BL21 (Novagene) for expression of wild-type and mutant RNase A.

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2.1. Mutagenesis and production of proteins

The RNase A mutant, H119N, was obtained by site-directed mutagenesis according to the manufacturer's specifications (Promega). Briefly the DNA fragment containing the RNase A structural gene was subcloned into the pSELECT-1TM vector (Promega). The following primer was used to introduce the histidine to asparagine substitution: 5′-CCGTACGTGCCAGTTAACTTTGATGCTTCAGTG-TAG-3′.

This sequence was designed to introduce an AAC asparagine codon together with a *HpaI* restriction site. The mutant variant of RNase A was verified by DNA sequencing and subcloned into the pMEW2 expression vector [14]. A previously described system for RNase A expression [14], which includes the P_R promoter of *E. coli* phage λ under cI857 regulation, was used to produce the recombinant wild-type and mutant RNase A proteins in *E. coli*. Fusion to the phoA signal peptide directed the expressed ribonucleases into the cell periplasm. Recombinant wild-type RNase A and the H119N mutant were purified in two steps by cation exchange and reverse-phase chromatography [14]. Protein purity was determined according SDS-PAAG electrophoresis, followed by silver staining and reverse-phase chromatography and was estimated as 99%. The yields ranged between 20 and 40 mg of pure protein per litre of cell culture.

2.2. RNase activity assay

RNase activity was assayed at pH 8.0 in 120 μl of a buffer containing 0.1 M Tris-HCl, 0.1 M NaCl and 1.6 mg/ml of yeast ribonucleic acid (Serva). After incubation at 37°C for 15 min, 300 μl of 2-propanol was added to the reaction with vigorous mixing. The samples were incubated for 20 min at -20°C and then centrifuged for 10 min at $14000 \times g$. The supernatant was removed and diluted a 100-fold in water and the absorbance at 260 nm was measured. The absorbance of the supernatant was a linear function of added enzyme up to an A_{260} of at least 1.5. One unit of ribonuclease activity in a 120 μl reaction volume will produce alcohol-soluble oligonucleotides equivalent to a ΔA_{260} of 1.0 in 30 min at pH 7.5 and 37°C.

2.3. Kinetic measurements

Experiments were performed with an Uvicon 1800 Spectrophotometer (Kontron Instruments). Thermostated cells (25°C) were used in kinetic measurements. Substrate cleavage was monitored at 278 nm for poly(U) ($\Delta\varepsilon = 1.360$ M^{-1} cm^{-1}, pH = 6.0) [8] and at 250 nm for poly(C) ($\Delta\varepsilon = 1.360$ M^{-1} cm^{-1}, pH = 6.0) [11]. The cleavage of UpA was monitored with an adenosine deaminase-coupled assay [15] at 265 nm ($\Delta\varepsilon = 6000$ M^{-1} cm^{-1}) [11]. Enzyme concentrations were measured at 277.5 nm, $\varepsilon = 9800$ M^{-1} cm^{-1} [16], pH = 6.5-7.0. The kinetic assays were performed at pH 6.0 where k_{cat}/K_M appears to be maximal at 25°C in 0.1 M MES-Tris buffer, $I = 0.1$ M (NaCl). Substrate concentrations ranged from 5 μM to 0.7 mM, enzyme concentrations were 0.6–2 nM for poly(C), 9–30 nM for poly(U) and 1–7 nM for UpA. In the case of wild-type and recombinant RNase albumine (at the concentration 0.1 mg/ml) was added to the stock solutions of enzymes for the kinetic measurements. The values of V_{max} and K_M were determined from initial velocity data using the ENZPACK3 program [17].

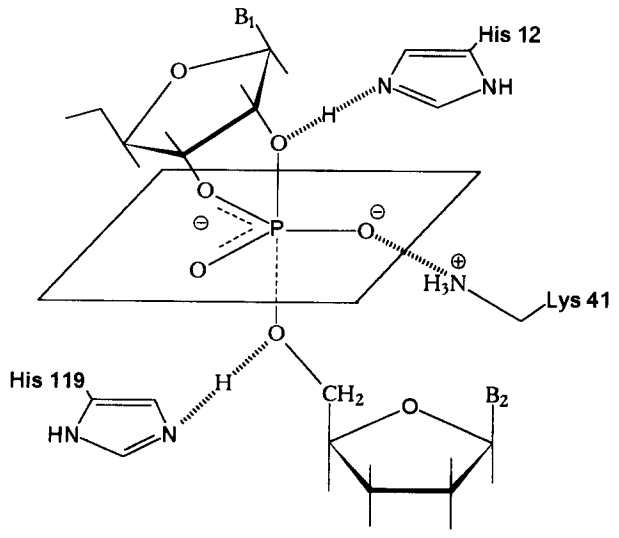

Fig. 1. Schematic representation of the transition state of the enzyme catalyzed reaction of RNase A.

3. Results and discussion

The use of asparagine at position 119 can provide information on the function of His^{119} in RNase A catalysis. Asparagine is a polar residue which is able to form hydrogen bonds with the phosphodiester bond of RNA (or other substrates), but it cannot donate a proton to a leaving group since the pK value of its carboxyamide group is close to 14. We carried out computer modelling to investigate possible effects of the mutation on the enzyme active site conformation and the structure of the enzyme–substrate complex. For this simulation the X-ray coordinates of a complex of RNase A with d(CpA) were used [4]. The side-chain of His^{119} is exposed and its mutation should not affect position and properties of active-site groups. In addition, the mutation should not disturb specific interactions betweeen the ligand and RNase, neither in the B_1 nor B_2 sites, with one obvious exception — the stacking interaction between the adenine and the imidazole-ring of His^{119} will be lost. In addition, the Asn side-chain could easily adopt an allowed conformation suitable for participation in hydrogen bonding with the ligand phosphate moiety. It was found that one of the allowed conformations for asparagine could form the corresponding hydrogen bond with the leaving O5′ oxygen of phosphate moiety (Fig. 2). In conclusion, the

Table 1
Steady-state kinetic parameters for cleavage of ribonucleotides by wild-type, recombinant ribonucleases and His^{119}Asn mutant

Substrate	RNase A	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})	Ratio[a]
poly(U)	Recombinant [11]	24 (15)	0.06 (0.01)	4 (3)×10^5	
	Wild-type	**9.1 (0.9)**	**0.049 (0.013)**	**1.9 (0.8)×10^5**	
	Recombinant	**10.3 (0.9)**	**0.076 (0.014)**	**1.4 (0.4)×10^5**	
poly(C)	Recombinant [11]	510 (10)	0.034 (0.002)	15 (1)×10^6	
	Recombinant	**603 (21)**	**0.052 (0.005)**	**11.5 (0.2)×10^6**	
	H119N	**4.37 (0.15)**	**0.047 (0.006)**	**9.3 (1.1)×10^4**	**8.1×10^{-3}**
UpA	Wild-type [18]	1920 (130)	0.33 (0.05)	5.8 (1)×10^6	
	Recombinant [11]	1400 (150)	0.62 (0.09)	2.3 (0.4)×10^6	
	Recombinant	**1170 (25)**	**0.40 (0.015)**	**2.9 (0.2)×10^6**	
	H119N	**15.5 (0.08)**	**0.44 (0.04)**	**3.6 (0.4)×10^4**	**1.24×10^{-2}**

[a] k_{cat}/K_M (H119N)/(k_{cat}/K_M (rRNase A)).
All reactions were performed at 25°C in 0.1 M MES buffer, pH 6.0, containing 0.1 M NaCl.
Experimental errors are in parentheses.

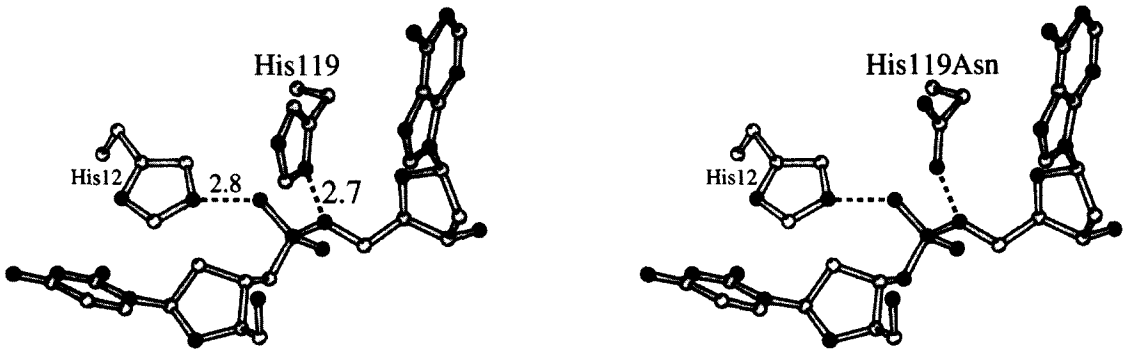

Fig. 2. A molecular model of the asparagine for histidine substitution in the active site of RNase A (complex with d(CpA) [4]).

H119N mutation is justified from a structural point of view: asparagine can participate in hydrogen bonding but it neither can donate/accept protons like histidine nor participate in stacking interactions with the nucleobase of a substrate leaving group. For this reason we constructed and characterized the H119N RNase A mutant.

The His^{119} to asparagine substitution was obtained using site-directed mutagenesis as described in Section 2. Protein expression was performed according to the protocol developed for wild-type RNase A with minor alterations [14]. It was found that heat-shock induction is more effective for production of the mutant protein when compared with wild-type RNase A. The cell culture containing BL21[pMEW2-H119N] was grown at 28°C and agitated at 220 rpm in a rich medium [14] to a density of approximately $OD_{600} = 0.6$ and then induced by heat shock to 42°C by addition of a half-volume of a prewarmed (85°C) rich medium. The culture was further agitated at 42°C and 120 rpm for 30 min. The temperature was reduced to 37°C and incubation was continued for a further 12 h. The level of mutant protein expression was similar to that of wild-type RNase A. Both proteins were purified using a previously developed purification scheme [14]. The periplasmic extract was adjusted to pH 6.0 and loaded onto a pre-equilibrated SP-Trisacryl column. The column was washed until the absorbance of the eluate at 280 nm became negligible. Target protein was eluted with 0.25 M ammonium acetate in 50 mM K,Na-phosphate buffer (pH 7.0). The fraction containing RNase activity (in the case of wild-type RNase A and a similar fraction in the case of mutant H119N) was loaded onto a C18 column. The column was washed and equilibrated with 0.1% TFA and the elution was performed with a linear gradient of 0-70% acetonitrile (with 0.07% TFA). The peak containing RNase activity (in case of the wild-type RNase A and a similar peak in case of the mutant H119N) was collected and freeze-dried. The protein yield for both wild-type and mutant RNase A was 75-80%. The catalytic properties of recombinant wild-type ribonuclease produced in our system towards different RNA substrates are close to those of bovine pancreatic RNase A and other recombinant RNases A (Table 1) [11–13,18,19].

We found that the relative RNase activity of the H119N mutant using RNA as substrate is severely reduced — less than 1% of wild-type RNase A activity. So we compared the catalytic properties of the purified H119N RNase A to those of recombinant wild-type RNase A obtained with the same expression system and to the properties of the H119A RNase A [13]. We were unable to measure the catalytic properties of the H119N mutant with poly(U) as a substrate under similar conditions used for poly(C) due to the unexpected problem, that the difference spectra of poly(U) and its products for the H119N mutant in the initial period of the reaction differed from that for wild-type RNase A.

If the catalytic role of His^{119} is restricted to transition state stabilization followed by protonation of a leaving group, then we would expect minor differences in K_M values between the wild-type and mutant enzyme and a substantial decrease in k_{cat} values with the H119N mutant for all substrates tested. These assumptions are supported by the fact that for RNase A (at the transesterification reaction) the K_M value is approximately equal to K_S [19]. The results presented in Table 1 indicate that K_M values for the cleavage of poly(C) and UpA by the H119N mutant are similar to those for wild-type RNase A. In contrast, k_{cat} values (for both substrates) decreased significantly. Thus, the k_{cat}/K_M values for mutant H119N decline by 100- to 1000-fold. This decrease is the same for both substrates used and are intermediate between those of wild-type RNase and mutant H119A (Fig. 3).

Our results indicate that the imidazole ring of His^{119} must be protonated in an enzyme-substrate complex to be able to contribute into the catalysis. This contribution is not restricted to protonation of a substrate leaving group oxygen atom and His^{119} participates directly in a transition state stabilization via hydrogen bonding.

The ratio of cleavage rate for poly(C) to UpA was not changed by the mutation and consequently one may assume

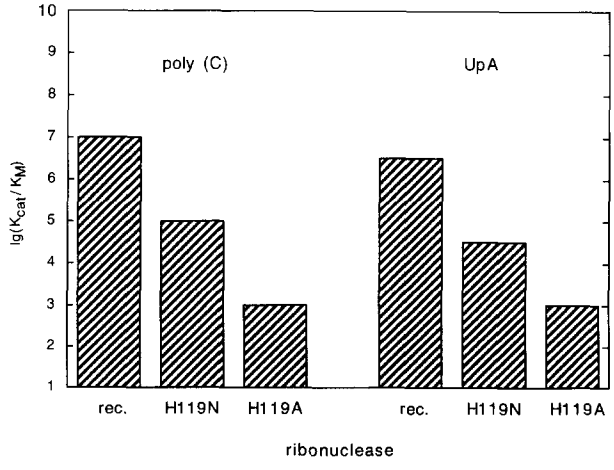

Fig. 3. Values of k_{cat}/K_M for the cleavage reaction catalyzed by recombinant, H119N and H119A [13] ribonucleases.

that the stacking interaction between the adenine moiety of UpA and the imidazole ring of His^{119} in the ground state does not explain the preference of RNase A for adenine in the leaving group. One may assume that the adenine ring of UpA in the complex of the mutant with UpA is kept in the same position as it was found in the complex with the native enzyme due to interactions with other groups present in the B_2 site [4]. These interactions occur with an enzyme molecule in which the imidazole ring of His^{119} is in the catalytically competent "A" conformation. Interactions of the adenine ring of UpA in the B_2 site and stacking interactions with the imidazole ring of His^{119} keep the latter in this "A" conformation.

The following conclusions regarding the mechanism of action of RNase A can be formulated on the basis of experimental results obtained with the H119N mutant:

1. The catalytic function of His^{119} is not confined to protonation of the leaving group in the phosphodiester cleavage reaction. The protonated imidazole ring of His^{119} also participates directly in transition state stabilization via hydrogen bonding with a substrate phosphodiester group.
2. His^{119} does not contribute much to the positioning of the adenine ring of UpA within the B_2 site of the RNase active center. Other interactions have a more important role, but these together with the stacking interaction between the adenine of UpA and the imidazole ring of His^{119} keep the latter in the catalytically competent "A" conformation. This means that "substrate assisted catalysis" makes a substantial contribution to the enhanced rate of UpA cleavage by native RNase A.

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