PROTON MAGNETIC RESONANCE STUDIES OF DES-(121–124)-RIBONUCLEASE A

V.G. SACHAROVSKY, I.I. CHERVIN and G.I. YAKOVLEV
Institute for Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences, Pushchino, USSR

S.M. DUDKIN, M.Ya. KARPEISKY* and S.V. SHLIAPNIKOV
Institute of Molecular Biology, USSR Academy of Sciences, Moscow, 117312, USSR

and

V.F. BYSTROV
Shemyakin Institute for Chemistry of Natural Products, USSR Academy of Sciences, Moscow, 117312, USSR

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1. Introduction

Bovine pancreatic ribonuclease A (RNAase A) has been extensively investigated from the point of view of structure and function [1].

NMR spectroscopy has been successful in providing information about the state of RNAase A in aqueous solution, estimates of the *pK* values for each histidine residue and an evaluation of the enzyme–inhibitor complex structure [2–5].

In this paper we report the PMR data for des-(121–124, Asp–Ala–Ser–Val–)-RNAase A, which was obtained by a limited hydrolysis of RNAase A and was designated as PIR [6–8].

The data presented here on the pH-dependence of PMR spectra of PIR and its complexes with cytidine-3′-phosphate (3′-CMP) were used to estimate the individual *pK*'s (apparent) for the four histidines in PIR as well as for a comparison of the conformations of both proteins.

The experimental data thus obtained suggest the role of Asp-121 in maintaining the precise orientation of the essential functional groups in the active site of RNAase A.

2. Materials and methods

Bovine pancreatic ribonuclease A (free of phosphate and dimers) was purified as described [9]. Pepsin inactivated ribonuclease A (PIR) was prepared as described by Lin [7]. The two proteins used were lyophilized from a 1% solution in D_2O after purification and were homogeneous when chromatographed on SE-Sephadex C-25 and Sephadex G-75.

The catalytic activity of PIR assayed with cytidine-2′, 3′-cyclophosphate as substrate was less than 0.5% of the native value. This residual activity was lost after treatment with carboxypeptidase-A [7, 8].

3′-CMP was prepared by enzymic hydrolysis of cytidine-2′, 3′-cyclophosphate. The UV and PMR spectra agreed well with those expected for 3′-CMP and showed no trace of 2′-CMP. All the other materials were reagent grade. All solutions were prepared in 98% D_2O and were 0.2 M in NaCl, 0.0064–0.0078 M in protein (RNAase A or PIR) and contained various amounts of 3′-CMP.

The pH was measured before and after running the spectrum directly in the NMR test-tube by means of specially made electrodes connected to the pH-meter (LPU-01, USSR).

Both readings agreed to within 0.04 pH units. Values of pH given are uncorrected glass-electrode meter readings and *pK* values were calculated from these fig-

* To whom all correspondence should be addressed.

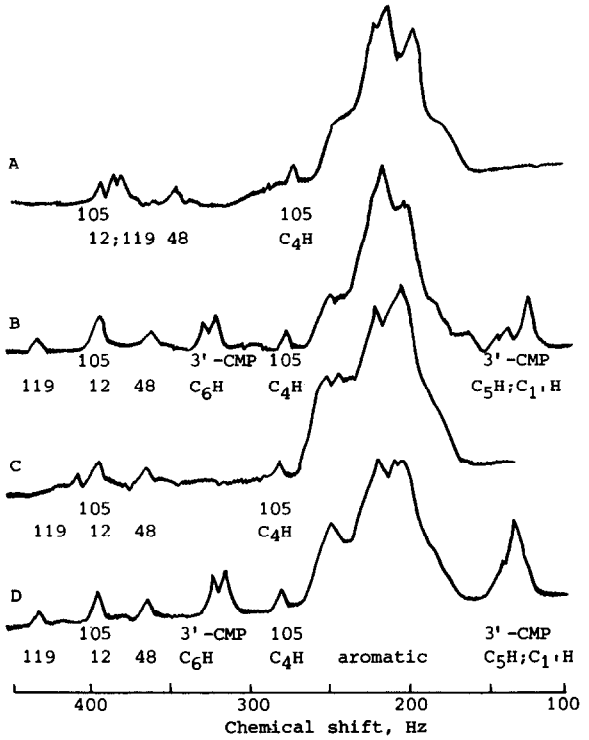

Fig. 1. 100 MHz PMR spectra of the histidine, phenylalanine and tyrosine absorption regions of 0.0064 M PIR and of 0.0073 RNAase A in presence or absence of 0.026 M 3'-CMP at pH 5.5. A) RNAase alone; B) RNAase + 3'-CMP; C) PIR alone; D) PIR + 3'-CMP.

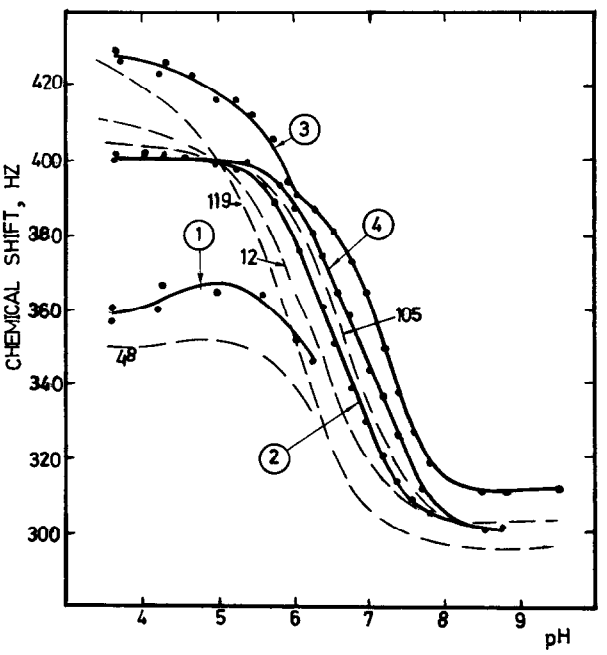

Fig. 2. Titration curves of His peaks of 0.0064 M PIR. The ordinate is the chemical shift of the C_2H peak in Hz (at 100 MHz), downfield from HOD. Dashed lines show titration curves of His peaks in RNAase A.

ures; the apparent *pK* values thus obtained are applicable to H_2O as well as to D_2O [10].

The PMR spectra were obtained at 100 MHz on a Varian HA-100D spectrometer at 34 ± 0.5°C, using a residual HOD peak in D_2O solution as an internal lock. Reported shifts are given in Hz relative to the water peak (HOD).

The spectra were recorded without accumulation, using sweep rate 0.4 Hz/sec and 0.2 Hz response.

3. Results and discussion

According to published data, the catalytic activity of PIR was only 0.5% of that of native RNAase, while its ability to specifically bind nucleotides was not changed markedly. The low catalytic activity of PIR was assumed to be due to the increased *pK* value of the His-119 residue. On the other hand, the change of this *pK* value might have resulted from the removal of the Asp-121 residue from the active site region [7, 8].

To clarify this point we studied the state of the histidines in des-(121–124)-ribonuclease A by the PMR technique. Several characteristic peaks are observed (fig. 1) throughout the histidine region of the PIR and the PIR–3'-CMP complex PMR spectra.

To assign the resonances in the aromatic region of the PMR-spectrum of PIR (C_2H histidine resonance) to the particular histidine residues in the protein molecule, the effect of pH and inhibitor binding were studied (figs. 2–4).

From fig. 2 it appears that more normal titration curves would be obtained if curves 3 and 4 crossed at pH 5.9. But in fact they do not cross. To prove this, PIR solution was saturated by 3'-CMP at pH 5.5 and 8.0. At both pH values the peak 3 moves downfield as a result of PIR–3'-CMP complex formation and its positions correspond to curve 3 on fig. 3. Consequently, the upper (pH 4–6) and the lower (pH 6–9) parts

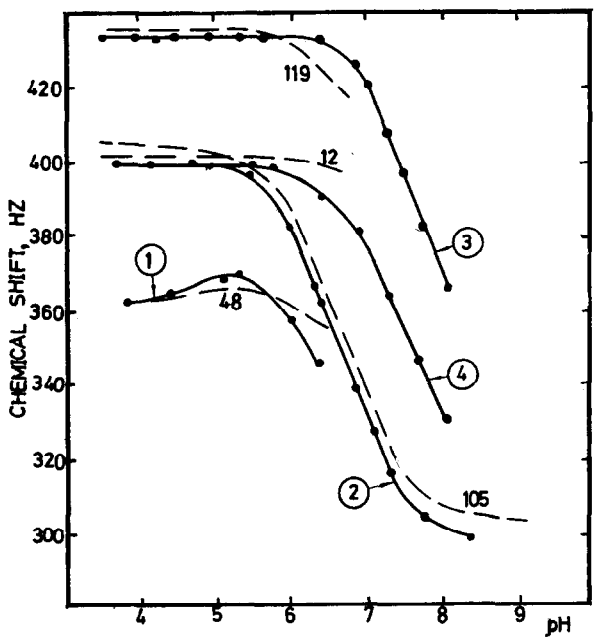

Fig. 3. Titration curves of His peaks in the PIR–3′-CMP complex. Concentrations are PIR, 0.0064 M; 3′-CMP, 0.065 M. Enzyme was saturated with inhibitor at pH 8.0. Dashed lines show titration curves of His peaks in the RNAase A–3′-CMP complex.

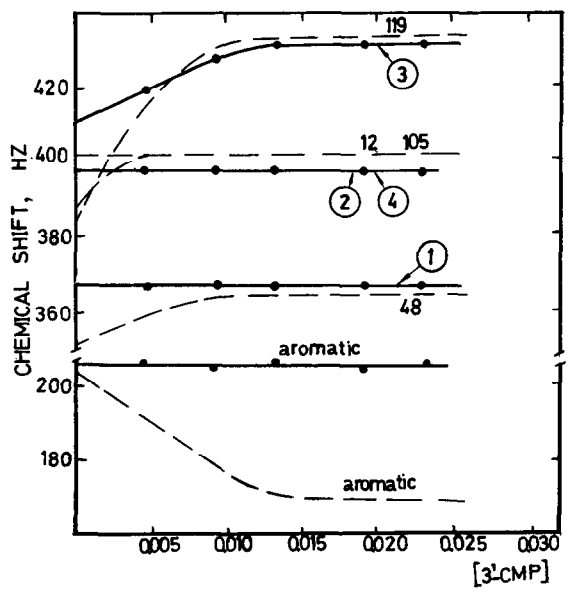

Fig. 4. Chemical shifts of PIR His peaks and main aromatic envelope as a function of the total concentration of added 3′-CMP at pH 5.5. Dashed lines indicate positions of the peaks of RNAase A under the same conditions. Concentrations of PIR and RNAase A are 0.0064 M.

of curve 3 on fig. 2 belong to the same titration curve. The "kink" at pH ~ 5.9 on this titration curve seems to reflect an interaction of corresponding His residue with neighbouring titratable PIR groups.

The individual *pK*'s values, listed in table 1, were estimated by differentiating the experimental titration curves of the protein histidine peaks, recorded in the presence and absence of 3′-CMP.

The comparison of PMR spectra of PIR and its complexes with 3′-CMP to those of RNAase A over the whole pH range studied clearly showed that curve 1 (figs. 2, 3) corresponds to His-48; curve 2, to His-105; curve 3, to His-119; curve 4, to His-12. The higher *pK* value of His-119 in comparison with His-12 is in agreement with the finding [8] that in PIR mainly His-12 is alkylated by iodoacetate at pH 5.5.

The experimental data obtained allow us to conclude that the local magnetic environment of His-48 and His-105 in PIR differs somewhat from their environment in RNAase A. To put it another way, there should exist some small conformational differences between PIR and RNAase A apart from the active site region. The downfield shift of the C_2H peak of protonated His-48 (fig. 2) should reflect a change in the relative orientation of the His-48 imidazole ring and of the Tyr-25 side chain [11].

In our opinion the main difference between the two proteins lies in the active site area. As is evident from the data listed in the table, the *pK* values of His-119 and His-12 of PIR increase by 1.3 and 0.4 pH units, respectively, as compared to those of RNAase A.

Table 1
Ionization constants for individual histidine residues.

Residue no.	PIR	RNAase A	PIR–3′-CMP	RNAase A–3′-CMP
119	7.1	5.8	7.5	7.5
12	6.8	6.2	7.4	7.9
105	6.6	6.7	6.7	6.7
48	(6.6)*	(6.6)*	–	–

* Estimated in the presence of the acetic acid.

The results obtained support the suggestion that the interaction between the distal carboxylic group of Asp-121 and N(3) atom of the His-119 imidazole ring should exist in the RNAase A solution. It would appear that it is this hydrogen bond which influences the *pK* value of His-119 of RNAase A and, furthermore, that this bond maintains the imidazole ring of His-119 in the catalytically active position. Consequently, the disruption of this hydrogen bond could give rise to the drastic decrease in the catalytic activity of PIR compared to that of RNAase A.

Thus, the suggestion that the C-terminal tetrapeptide of RNAase A maintains the catalytic active configuration of the functional groups in the active site would appear to be confirmed.

Our data on the binding of 3′-CMP to PIR are in line with this conclusion. Fig. 4 shows the changes in the chemical shift of the imidazole ring C_2H, and the main aromatic resonances of PIR resulting from the inhibitor binding. There is a difference between RNAase A and PIR in that no upfield shift of the peak in the main aromatic envelope is observed in the latter case when 3′-CMP is added.

Thus, the mutual orientation of the cytosine ring of 3′-CMP in the PIR–3′-CMP complex must differ from that of the RNAase A–3′-CMP complex.

Moreover, C_2H of His-12 in the PIR–3′-CMP complex does not position itself in the same way as the C_2H of His-12 in the RNAase A–3′-CMP complex with respect to the 3′-CMP phosphate group.

This conclusion seems to be reasonable when one takes into account the lack of upfield shift of the His-12 C_2H resonance when comparing the fully protonated form and the PIR–3′-CMP complex (figs. 3, 4) Previously it has been shown that the His-12 C_2H peak shifted ~ 10 Hz upfield in fully protonated RNAase–3′-CMP complex [3].

It is of interest to note that by X-ray diffraction studies, the hydrogen bond under consideration has not been detected in the crystal structure of RNAase S [1].

Since RNAase A and RNAase S have approximately the same catalytic activity in solution, one can assume that the active site conformation of RNAase S in solution differs from that of the crystal form.

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