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^{1}H-NMR STUDIES ON THE BINDING SUBSITES OF BOVINE PANCREATIC RIBONUCLEASE A

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The titration curves of the C-2 histidine protons of an RNAase derivative (a covalent derivative obtained by reaction of bovine pancreatic RNAase A (EC 3.1.27.5) with 6-chloropurine 9-β-D-ribofuranosyl 5′-monophosphate) were studied by means of ^{1}H-NMR spectroscopy at 270 MHz. The interaction of natural (5′AMP, 5′GMP, 5′IMP) and halogenated purine mononucleotides (cl^{6}RMP, br^{8}AMP) with RNAase A was also monitored by using the same technique. The slight change observed in the p*K* values of the active centre histidine residues of the RNAase derivative, with respect to those in the native enzyme, can be considered as evidence that the phosphate of the label does not interact directly either with His-12 or 119 in the p_1 site, but in the p_2 site as proposed previously (Parés, X., Llorens, R., Arús, C. and Cuchillo, C.M. (1980) Eur. J. Biochem. 105, 571–579). Lys-7 and/or Arg-10 are proposed as part of the p_2 phosphate-binding subsite. The p*K* values of His-12 and 119 and the shift of an aromatic resonance of the native enzyme found on interaction with some purine nucleotides, can be interpreted by postulating that the interaction of 5′AMP, 5′GMP and 5′IMP takes place not only in the so-called purine-binding site $B_2R_2p_1$ but also in the primary pyrimidine-binding site B_1R_1 and p_0 of RNAase A.

Introduction

Extensive work on the nature of the sites responsible for the interaction between RNAase A (EC 3.1.27.5) and its substrate ribonucleic acid, has been done mainly through the use of X-ray diffraction [1–4] and NMR [5–9] methods with model compounds. These works together with affinity labelling studies [10], show the existence of at least three phosphate-binding sites called p_0, p_1 and p_2 and two sites for the binding of the nucleoside moieties namely B_1R_1 and B_2R_2. Moreover, the early kinetic work of Witzel and colleagues revised by Richards and Wyckoff [1], show that there is a clear selectivity in the rate of hydrolysis of dinucleoside monophosphates depending on the nucleoside present in the 5′ position, the order being A > G > C > U.

The aim of the present work was to further characterize p_2, the phosphate-binding subsite adjacent to the 5′ position of the bond cleaved. This was achieved by studying the perturbation of the active centre histidine residues of derivative II, a covalent derivative of RNAase A with the halogenated nucleotide cl^{6}Pur-RibP [10]. To this end, the titration curves of the histidine C-2 protons were followed by means of high resolution ^{1}H-NMR spectroscopy at 270 MHz. In addition to this, the interactions between several purine nucleotides and native

Abbreviations: cl^{6}Pur-RibP, 6-chloropurine 9-β-D-ribofuranosyl 5′-monophosphate; br^{8}AMP, 8-bromoadenosine 5′-monophosphate; 2′-FdUrd-P-Ado, 2′-deoxy-2′-fluoridyl (3′–5′)adenosine; C2′–5′A, cytidylyl-(2′–5′)adenosine; derivative II, a covalent derivative of RNAase A and cl^{6}Pur-RibP with the bond established between the α-NH_2 of Lys-1 and the C-6 of the purine ring.

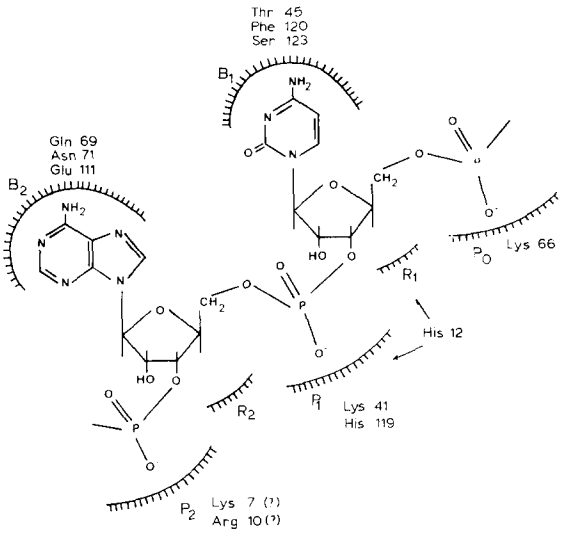


Fig. 1. Schematic illustration of the binding of an RNA fragment to RNAase A. In each subsite are listed the amino acids the participation of which is known or postulated. $B_1R_1p_1R_2B_2$ is the region occupied by the substrate analogues Up[CH_2]A [1] and C2'–5'A [3]. The cleavage takes place at the P-05' bond of the phosphate occupying p_1. 5'AMP binds preferentially to $B_2R_2p_1$ and 3'CMP to $B_1R_1p_1$ [1]. p_2 would be the site for the phosphate in the RNAase A-3'AMP complex and would bind the phosphate of cl^{6}Pur-RibP when it reacts with Lys-1 to yield derivative II [10].

RNAase A were studied. This work was undertaken in order to better understand the specific relationships between the nucleoside moieties of the substrate and the corresponding binding regions in the enzyme.

In order to interpret the results found in the present work a careful review of the vast amount of NMR data published before the reassignment of the resonances of the active centre histidine residues [11,12] was necessary.

Materials and Methods

RNAase (twice crystallized) was from Cambrian Chemicals (Croydon, Surrey, U.K.). This material was purified by the method of Taborsky [13] to obtain the RNAase A fraction. 5'AMP-Na_2 was from Merck (Darmstadt, GFR). 5'GMP-Na_2 was from Koch-Light Lab. (Colnbrook, Bucks, U.K.), and 5'IMP-Na_2 · $7H_2O$ was from Calbiochem (Los Angeles, CA, U.S.A.). cl^{6}Pur-RibP-Ba and br^{8}AMP-Na_2 were synthesised as described earlier [14]. NaCl, analytical grade was from Merck, C^2H_3COONa was obtained by reaction of equimolar amounts of $C^2H_3COO^2H$ (Aldrich, Milwaukee, WI, U.S.A.) and NaO^2H (Aldrich) followed by repeated freeze-drying from ^2H_2O (99.98% isotopical purity) (Aldrich).

The exchangeable protons of the protein samples were deuterated following the procedure of Patel et al. [15]. Final solutions of RNAase A (60 mg · ml^{-1}) were made in ^2H_2O containing 0.2 M NaCl or C^2H_3COONa, to which the desired amount of nucleotide was added. Except for the saturation curves, the ligand/enzyme molar ratio was always 6 : 1. In the case of derivative II, a lower protein concentration (24 mg · ml^{-1}) was used. The pH was measured in the NMR sample tube with a special combined electrode (Microelectrodes Inc., Londonderry, NH, U.S.A.) and adjusted to the desired value by adding 1 M ^2HCl (Aldrich). For fine adjustments, 0.1 M ^2HCl solutions were used. All pH measurements, done before and after recording the spectra, were direct meter readings. They were accepted only if they agreed to within 0.04 pH units.

A 270 MHz Bruker WH-270 spectrometer, installed at the 'Centro di Metodologie Chimico-Fisiche' of the University of Naples, was used and the NMR spectra were usually obtained by accumulating 500 scans, except in the case of derivative II in which longer accumulating periods were needed. Chemical shifts are reported in parts per million (ppm) downfield from internal $(CH_3)_3Si(CH_2)_3 \cdot SO_3Na$ at an ambient probe temperature of 32 ± 2°C.

Curve fitting was carried out by using a program of least-squares iterative regression fit [16], adjusted for its use in an UNIVAC 1108 computer. The following formulae [17] were used:

(a) For a simple proton association-dissociation equilibrium:

$$\delta_{obs} = \delta_0 + \frac{\Delta \cdot 10^{(pK-pH)}}{1 + 10^{(pK-pH)}} \quad (1)$$

where δ_{obs} is the observed chemical shift, δ_0 is the chemical shift of the unprotonated form and Δ is the chemical shift change upon protonation.

(b) In the cases where an acid inflection was present the equation used was:

$$\delta_{obs} = \delta_0 + \frac{\Delta_1 \cdot 10^{(pK_1-pH)}}{1 + 10^{(pK_1-pH)}} + \frac{\Delta_2 \cdot 10^{(pK_2-pH)}}{1 + 10^{(pK_2-pH)}} \quad (2)$$

where Δ_1 and Δ_2 are the chemical shift changes for the acid pH inflection (pK_1) and the basic pH inflection (pK_2), respectively.

Results and Discussion

The experiments reported in this paper were carried out in 0.2 M sodium acetate solutions in which a full titration curve of His-48 can be observed in contrast to NaCl solutions where this resonance broadens and disappears above pH 5 [18,19]. It had been shown [20,21] that His-12, 105 and 119 have, essentially, the same pK values in both NaCl and sodium acetate. This point was checked again in this work. Moreover, the pK values of RNAase A in the presence of 5'AMP in NaCl [6,8] also agree with those found in sodium acetate (Tables I and II). No significant changes were observed upon addition of ligands to the native enzyme, or in derivative II, in the pK of His-105. As it is seen in Table I and Figs. 2 and 3, a better fit of the calculated curve to the experimental points can be obtained for the titration curves of His-12, 48 and 119 if the existence of an acid inflection is accepted. Therefore, two pK values are reported except in those cases where no acid inflection can be observed. The large error for pK_1 values is due in all cases to the uncertainty in the experimental points which is close to the width of the acid inflection. It had been postulated [20,22], that either Asp-14 or 121 could be responsible for the acid inflection of His-12, 48 and 119. However, recent work by Niu et al. [23] shows that any interaction between His-12 and Asp-14 is unlikely. In the case of the interaction with purine nucleotides an acid inflection is also observed. The pK_1 values can be estimated although their large uncertainties do not allow any useful comparison with other values reported in the literature.

1. Chemical shift data

The identification of the individual histidine resonances in the titration curves of RNAase A and of RNAase A + 5'AMP was done according to Patel et al. [11] and Haffner and Wang [6], respectively, although in the latter case a reversed assignment for His-12 and 119 [11,12] was assumed. For derivative II where small changes in chemical shifts are observed (Fig. 2) the same assignments were used. For the

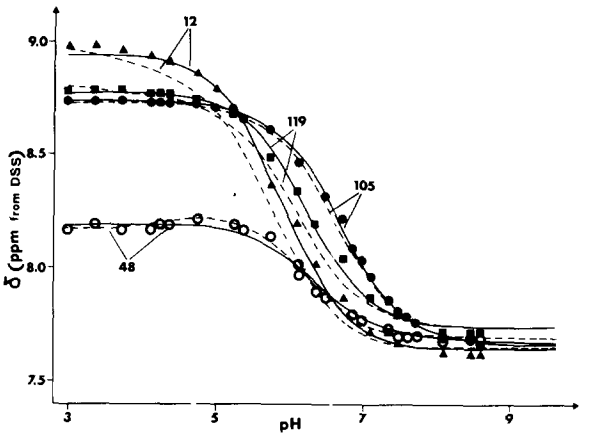


Fig. 2. pH dependence of the chemical shifts of the histidine C-2 proton resonances of derivative II. For comparison the histidine titration curves of the free enzyme are also included (dashed lines). Numbers refer to the histidine residues in the sequence of RNAase A. Conditions: 1.7 mM derivative II in 0.2 M sodium acetate. ▲, His-12; ○, His-48; ●, His-105; ■, His-119.

other experiments, saturation curves at constant pH were needed to unambiguously identify the individual resonances (data not shown). In the experiments run in the presence of 5'GMP, 5'IMP and 5'AMP, the shift of an aromatic resonance affected by the binding of these nucleotides is also shown. The halogenated nucleotides cl^{6}Pur-RibP and br^{8}AMP did not produce any appreciable effect on such resonance. This aromatic resonance has an area corresponding to two protons and the chemical shift reached after saturation is, in all cases, slightly upfield (0.1–0.2 ppm) as compared to that observed on interaction of pyrimidine nucleotides with the enzyme [32]. This resonance was first assigned to Phe-120 [5,24], then to Tyr-25 [33] and lately reassigned again to Phe-120 [32,33]. The shift observed in the case of 5'AMP does not agree with the results of Haffner and Wang [6] and of Haar et al. [8]. The discrepancy can be attributed, in the former case, to the lower resolution (100 MHz) and higher ligand concentration ([L]/[E] = 15 : 1) as the ribose H 1' resonance is very close to the aromatic signal. In the latter case, [8], the ligand/enzyme ratio (1.9 : 1) is below saturation and this could undoubtedly affect the results.

TABLE I

APPARENT pK VALUES OF THE HISTIDINE AND OTHER RESIDUES OF RNAase A ALONE, DERIVATIVE II AND RNAase A-NUCLEOTIDE COMPLEXES

When two pK values are given for a single titration curve, the Eqn. 2 assuming an imidazole-carboxyl group interaction as explained in Materials and Methods, was used. In the other cases Eqn. 1 was used.

Nucleotide or derivative	His-12		His-119		His-48		His-105	Groups influencing chemical shift of the aromatic resonance	
	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2	pK	I	II
None [a]	–	5.83 ± 0.02	–	6.13 ± 0.02	–	–	6.67 ± 0.02	–	–
None [b]	–	5.74 ± 0.02	–	6.09 ± 0.03	4.66 ± 0.41	6.14 ± 0.10	6.65 ± 0.03	–	–
None	4.17 ± 0.43	5.83 ± 0.04	3.78 ± 0.80	6.13 ± 0.04	4.66 ± 0.41	6.14 ± 0.10	6.65 ± 0.03	–	–
Derivative II	–	5.89 ± 0.02	–	6.26 ± 0.03	5.18 ± 0.9	6.16 ± 0.2	6.71 ± 0.02	–	–
5'GMP	4.08 ± 0.19	7.51 ± 0.03	3.56 ± 0.37	7.57 ± 0.03	3.47 ± 1.07	6.33 ± 0.06	6.77 ± 0.02	3.87 ± 0.19	7.68 ± 0.09
5'IMP	4.27 ± 0.23	7.11 ± 0.03	4.32 ± 0.32	7.14 ± 0.04	3.91 ± 1.01	6.35 ± 0.05	6.76 ± 0.02	4.12 ± 0.16	7.50 ± 0.07
5'AMP	4.50 ± 0.23	6.52 ± 0.04	4.31 ± 0.41	7.57 ± 0.03	4.74 ± 0.81	6.28 ± 0.10	6.77 ± 0.02	4.38 ± 0.11	7.70 ± 0.16
cl[6] Pur-Rib P	4.49 ± 0.13	6.47 ± 0.03	4.28 ± 0.14	6.84 ± 0.03	4.35 ± 0.68	6.20 ± 0.07	6.76 ± 0.02	–	–
br[8] AMP	4.21 ± 0.21	6.33 ± 0.04	4.27 ± 0.34	8.03 ± 0.03	4.48 ± 0.63	6.19 ± 0.07	6.71 ± 0.02	–	–

[a] Titration carried out in 0.2 M NaCl. All other experiments were in 0.2 M sodium acetate.
[b] The fit was obtained by using Eqn. 1 for His-12, 119 and 105 and Eqn. 2 for His-48.

TABLE II

SUMMARY OF APPARENT pK VALUES OF THE HISTIDINE RESIDUES OF FREE RNAase A, DERIVATIVES AND THEIR COMPLEXES WITH NUCLEOTIDES OR NUCLEOSIDES, FOUND IN LITERATURE.

When an acid inflection has been considered its pK value is given in parentheses. [L]/[E_0] is the [Ligand]/[Enzyme] molar ratio. Up [CH_2]A is the phosphonate analogue of UpA in which the oxygen atom of the phosphoester bond between the phosphate and the adenosine moiety is replaced by a Z-CH_2-Z group.

Ligand or derivative	Salt	[L]/[E_0]*	pK				Reference
			His-12	His-119	His-48	His-105	
None	0.2 M NaAc	–	5.8	6.2	6.4	6.7	[21]
None	0.2 M NaCl	–	5.70	6.23	–	6.65	[18]
None	0.2 M NaCl	–	5.8	6.1	–	6.5	[24]
None	0.1 M NaCl	–	6.1	6.2	–	6.7	[25]
None	0.2 M NaCl	–	5.8	6.2	–	6.7	[26]
None	0.2 M NaCl	–	5.8	6.3	–	6.7	[27]
None	0.2 M NaCl	–	5.8	6.3	–	6.58	[6]
None	0.1 M NaCl	–	6.05 ± 0.05 (4.61 ± 0.15)	6.31 ± 0.05 (5.01 ± 0.39)	–	6.76 ± 0.02	[28]

None	0.2 M NaAc	–	6.06 ± 0.05 (4.11 ± 0.16)	6.35 ± 0.05 (4.12 ± 0.30)	6.31 ± 0.08	–	[20]
None	0.2 M NaCl	–	6.04 ± 0.03 (4.49 ± 0.12)	6.15 ± 0.05	–	6.60 ± 0.02	[17]
Inorganic phosphate	0.1 M NaCl	13.7	7.15 ± 0.02 (4.33 ± 0.05)	7.63 ± 0.05 (4.73 ± 0.21)	–	6.96 ± 0.04	[28]
Cytidine	0.2 M NaCl	12.3	6.4	6.2	–	6.7	[5]
5-Methyl-2′-deoxycytidine	0.2 M NaCl	12.3	6.2	6.2	–	6.7	[5]
3′CMP	0.2 M NaCl	4.6	7.4	8.0	–	6.7	[5]
3′CMP	0.2 M NaCl	1	7.5	7.9	–	6.7	[26]
2′-deoxycytidine-3′-phosphate	0.2 M NaCl	1	6.5	8.0	–	6.7	[8]
2′CMP	0.2 M NaCl	6.2	>8.0	8.0	–	6.7	[5]
Cyt-*N*(3)-oxide-2′-phosphate	0.2 M NaCl	1	Exchange broadening	7.9	–	6.7	[8]
5′CMP	0.2 M NaCl	–	<7.0	8.0	–	6.7	[5]
3′UMP	0.2 M NaCl	1.2	6.2	8.0	–	6.7	[8]
2′UMP	0.2 M NaCl	1.1	6.3	7.8	–	6.7	[8]
2′-FdUrd-3′-phosphate	0.2 M NaCl + 0.05 M NaAc	10	7.31 ± 0.04 (2.88 ± 0.14)	7.81 ± 0.04	6.33 ± 0.06 (3.00 ± 0.45)	6.72 ± 0.04	[9]
5′AMP	0.2 M NaCl	15.6	6.3	7.6	–	6.58	[6]
5′AMP	0.2 M NaCl	1.9	6.3	7.6	–	6.8	[8]
3′AMP	0.2 M NaCl	1.8	6.0	6.0	–	6.7	[8]
2′AMP	0.2 M NaCl	1.8	6.3	abnormal curve	–	6.7	[8]
8-oxoadenosine-3′-phosphate	0.2 M NaCl	1	6.6	7.3	–	6.6	[8]
8-oxoadenosine-3′-phosphate	0.2 M NaCl + 0.05 M NaAc	4	6.8	7.8	6.4	6.7	[29]
Up[CH_2]A	0.1 M NaCl	3	5.8	6.1	–	–	[30]
2′-FdUrd-P-Ado	0.2 M NaCl + 0.05 M NaAc	10	6.60 ± 0.06 (3.59 ± 0.12)	6.71 ± 0.04	6.38 ± 0.06 (3.60 ± 0.24)	6.71 ± 0.04	[9]
2′-FdUrd-P-(Me)	0.2 M NaCl + 0.05 M NaAc	10	6.47 ± 0.05 (3.67 ± 0.13)	6.70 ± 0.04	6.37 ± 0.06 (3.50 ± 0.28)	6.73 ± 0.04	[9]
EDTA	0.2 M NaAc	30	6.73 ± 0.02	6.08 ± 0.03	6.23 ± 0.05	6.69 ± 0.01	[31]
PIR *	0.2 M NaAc	–	7.1	6.8	–	6.6	[26]
PIR + 3′CMP	0.2 M NaAc	10	7.5	7.4	–	6.7	[26]

* PIR is des(121-124, Asp-Ala-Ser-Val-)-RNAase A.

The titration curves observed in the presence of cl^{6}Pur-RibP, always show an acid inflection at low pH values. This behaviour had already been observed by Cohen et al. [28] on interaction of RNAase A with saturating amounts of inorganic phosphate. It is interesting to note that the acid inflection is accompanied by an upfield shift (0.2–0.4 ppm) in the titration curve of His-119 when the enzyme interacts with cl^{6}Pur-RibP. This shift is similar to that observed for 3′AMP [8]. On the other hand no shift is observed upon interaction of the enzyme with 5′AMP, br^{8}AMP and 8-oxoadenosine 3′phosphate [8], and only a small shift (0.1 ppm) for inorganic phosphate [20] and 5′GMP and 5′IMP below pH 5. It is not possible, at present, to provide any explanation for these trends since the interaction of His-119 with its surroundings depends on various factors such as the phosphate location in p_1 or p_2 and the nature, orientation and conformation of the heterocyclic rings. In addition, further interactions between His-119 and Asp-121 in the free enzyme have also been postulated [2,9,20,34,35].

With respect to purine nucleotides it can be seen that both the pK values of His-12 and the shapes of the titration curves (Fig. 3) clearly differ from those of the enzyme alone. The C-2 proton of His-12 presents a downfield shift all along the curve, which is more evident at acid pH (0.4–0.5 ppm) in the cases of 5′GMP and 5′IMP. This shift is absent in the cases of 5′AMP, cl^{6}Pur-RibP and br^{8}AMP, or even replaced by a slight upfield shift. A downfield shift of a smaller magnitude has been found when a cytidine nucleotide interacts with RNAase A [5,8,18,24]. With uridine nucleotides it is not only absent but converted to an upfield shift [8]. In addition, there is an extensive broadening of the His-12 resonance at neutral pH with 5′GMP and, to a lower extent, with 5′IMP. This effect which has not been observed with 5′AMP, cl^{6}Pur-RibP and br^{8}AMP may reflect a slow chemical exchange between two or more different sites.

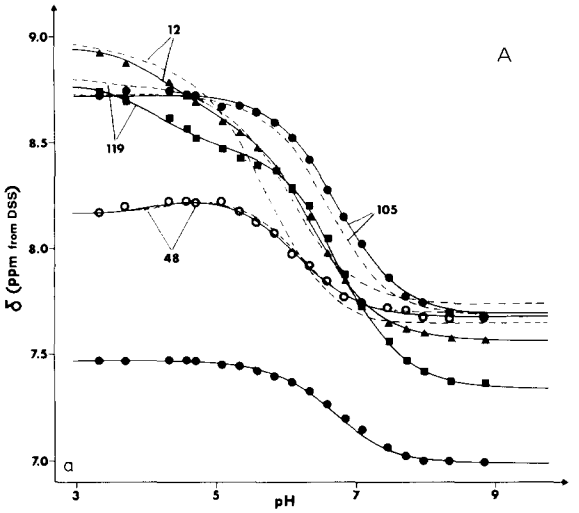
Another important feature to be considered, mainly with 5′GMP, 5′IMP and 5′AMP, is the shift of an aromatic resonance further upfield than that observed upon interaction of pyrimidine nucleotides [32]. This is the effect that could be expected if a ring with a π electron density higher than that of a pyrimidine, interacts with two of the ring protons of Phe-120. In analogy to the study of the interaction of 2′-FdUrd-P-Ado with RNAase A [9] a downfield inflection of the aromatic resonance assigned to Phe-120 at acidic and basic pH values (Table I) is also observed. Antonov et al. [9] postulated that this behaviour would reflect the dissociation of the ligand-enzyme complex that occupies in their case $B_1R_1p_1R_2B_2$. Since the perturbation of Phe-120 is closely associated with the binding of the base to B_1 [6,9], the similar behaviour observed when 5′GMP, 5′IMP and 5′AMP interact with RNAase A suggests that these purine nucleotides can bind not only in the $B_2R_2p_1$ site but also in $B_1R_1p_0$, at least at the ligand/enzyme molar ratio of 6 : 1 used in the present experiments.

The downfield shift of the titration curve of His-48 should also be considered. It is present with 5′GMP and 5′IMP to the same extent as with cytidine nucleotides (0.08–0.1 ppm) or uridine nucleotides (0.07 ppm) and it is absent in the case of 5′AMP, br^{8}AMP and cl^{6}Pur-RibP. The magnitude of this shift was interpreted by Haar et al. [8] as a measure of the strength of the interaction of the pyrimidine base in B_1 with the Phe-120 ring. The shift is also present, at pH 5.8, upon interaction of RNAase A with oxoadenosine 3′-phosphate [29], a nucleotide that binds to $B_1R_1p_1$ [8,29]. The interaction of the pyrimidine nucleoside in B_1R_1 would affect, through a conformational change, the interaction His-48-Asp-14 [20,22,23,32] producing the observed downfield shift. According to all the above considerations, the strength of the interaction in the B_1R_1 site would follow approx. 5′GMP > 5′IMP > 5′AMP. The halogenated nucleotides cl^{6}Pur-RibP and br^{8}AMP do not seem to appreciably interact in B_1R_1. The chemical shift together with the pK data (next section) show that the behaviour of 5′GMP and 5′IMP is similar to that of cytidine nucleotides, whereas that of 5′AMP would be similar to that of uridine nucleotides.

2. Dissociation constants of the histidine residues

The pK_1 and pK_2 values for the dissociation of the histidine imidazole rings are reported in Tables I and II. Before discussing them, some important points should be mentioned.

In the native enzyme, His-12 and 119 show abnormally low pK values. It is generally accepted [1,36], that this is due to the positively charged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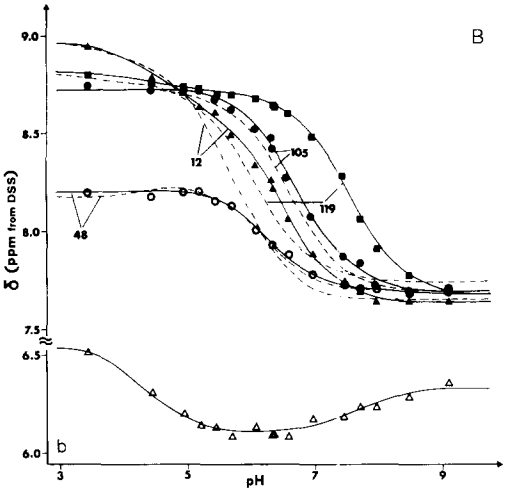
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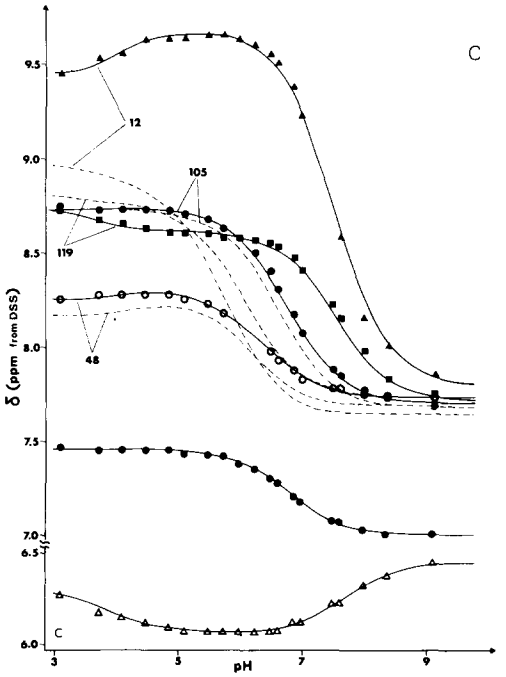
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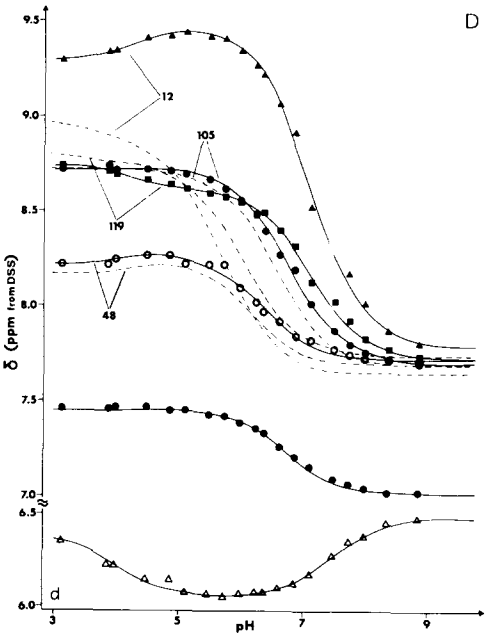
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pH

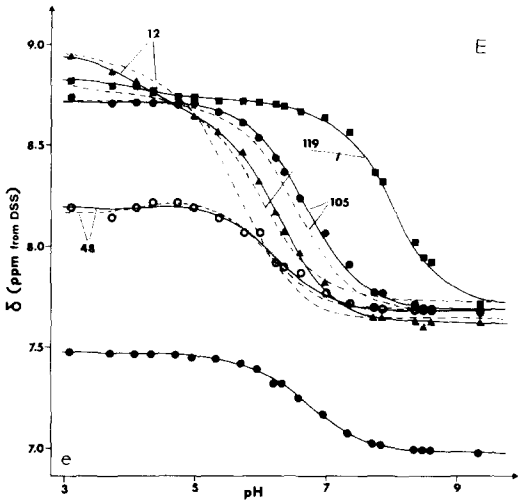

Fig. 3. pH dependence of the chemical shifts of the histidine C-2 proton resonances of RNAase A in the presence of (A) cl^{6}Pur-RibP, (B) 5'AMP, (C), 5'GMP, (D) 5'IMP, and (E) br^{8}AMP. In some of them, the chemical shift of the imidazole C-4 proton resonance of His-105 is also included. For comparison the histidine titration curves of the free enzyme are also shown (dashed lines). Numbers refer to the histidine residues in the sequence of RNAase A. Conditions. 4.38 mM RNAase A in 0.2 M sodium acetate. The ligand/enzyme molar ratio was 6 : 1 in all cases. ▲, His-12; ○, His-48; ●, His-105; ■, His-119; △, Phe-120. DSS, $(CH_3)_3Si(CH_2)_3 \cdot SO_3Na$.

cluster of lysine and arginine residues (Lys-7, 41 and 66 and Arg-10 and 39) near the primary phosphate-binding site p_1. The binding of pyrimidine competitive inhibitors such as 2'CMP or 3'CMP in $B_1R_1p_1$ increase both pK_2 values up to 7.5–8.0 [5,26]. X-Ray diffraction studies [2] have postulated that His-12 not only interacts with the phosphate in p_1, but also with the 2'OH of the ribose located in R_1. This hypothesis is confirmed by the pK value of His-12 (6.5 vs. 7.5) when the ribose 2'OH is replaced by a proton [8] and by the pK increase found when a phosphate (as in the case of 2'CMP) or a halogen replace the 2'-hydroxyl group [5,9]. Furthermore, cytidine by itself affects the pK of His-12 but not that of His-119 [5]. On the other hand, the imidazole ring of His-119 is strongly affected by the presence of a phosphate in p_1 and it is thought that four different orientations of the ring within the active site, denoted positions I to IV [1], are possible. When a purine ring is located in B_2, His-119 adopts position IV and as a result the interaction between His-119 and Asp-121 ([1–3,9,26,34] and Ref. 35 and references therein) could be weakened and the pK_2 value of His-119 would decrease correspondingly. A clear demonstration of this phenomenon is illustrated in the experiments carried out with pepsin-inactivated RNAase (Table II) where, in the presence of 3'CMP, the pK_2 value of His-119 is lowered with respect to native RNAase A (7.4 vs. 7.8–8.0). Moreover, in comparison with 2'CMP or 3'CMP the interaction of inorganic phosphate alone lowers the pK_2 value of His-119 [28] showing that the pyrimidine rings are effective in orienting the histidine ring towards Asp-121.

Pyrimidine nucleotides do not always affect the behaviour of histidine residues to the same extent. Considering that the high perturbation of both His-12 and 119 is mainly due to the strength of binding in $B_1R_1p_1$, cytidine monophosphates (2' and 3') appear to be strongly bound (pK_2 values are high for both histidines), whereas uridine 2' or 3' monophosphates appear to be less strongly bound (His-12 has a rather low pK_2 value and His-119 a value as high as in the cytidine nucleotides) (see Table II).

On the basis of these considerations the effect of the inhibitors used in the present work can now be examined.

a. Derivative II. A comparison between the titration curves of RNAase A and derivative II, shows that there is only a slight perturbation of the pK values of the active centre histidine residues (Table I, Fig. 2). On the other hand, if the effect produced on His-12 and 119 by the interaction of cl^{6}Pur-RibP (Table I), the reactant used in the synthesis of derivative II, is considered, it is then clear that the higher perturbation of the pK values of the active site histidine residues upon interaction with the nucleotide is due to the phosphate group being located in a position closer to the phosphate-binding site p_1. These results show that the phosphate group in derivative II lies far from the enzyme catalytic centre, and lends support to the hypothesis about the existence of p_2 [10]. Moreover, no perturbation of the aromatic resonance could be monitored.

Model building shows that the phosphate of the labelling nucleotide in derivative II is at the right distance from the α-NH_2 group of Lys-1 (to which it is linked [10,37]) to interact with Lys-7 and/or Arg-10 making them likely candidates to be part of the phosphate-binding site p_2 (Fig. 1). The possible role

of Lys-7 as part of the phosphate-binding site p_2 is supported by the fact that RNA protects Lys-7 against acetylation by acetic anhydride significantly more than any other lysine residues, Lys-41 included, whilst 3'CMP, which would not interact in p_2, does not show this effect [38]. In addition, both Lys-7 and Arg-10, are invariant residues in the pancreatic ribonucleases of 24 species tested [39]. The interaction of the phosphate of the labelling nucleotide with Lys-7 and/or Arg-10 would slightly alter the cationic cluster around His-12 and 119, thus explaining the small increase in their pK values. As His-119 in the base form is known to be essential in the second step of the catalysis [1,40–42] an increase of its pK value should slightly favour the catalitically inactive form at the pH considered. Thus, the small increase in the pK of His-119 (approx. 0.2 pH units) in derivative II, with respect to the native enzyme, would be in agreement with the decrease in k_{cat} (while K_m remains constant) measured in the kinetics of derivative II using cytidine 2',3'-phosphate as substrate [10], provided that the perturbation is maintained in the enzyme-substrate complex.

There is a close similarity between the pK values obtained by Haar et al. [8] (Table II) for the binding of 3'AMP with RNAase A and those found for derivative II. His-12 has the same pK_2 value in both cases (6.0) and the pK_2 values for His-119 are very similar (6.3 and 6.0). These results can be simply explained if the phosphate of 3'AMP (at the ligand/enzyme molar ratio of 1.9 : 1 used by Haar et al. [8]) interacts in the p_2 site too, as proposed by Parés et al. [10], thus causing the same effect on the cationic cluster around the active centre histidine residues. The lower pK_2 value (6.0 vs. 6.3) for His-119 in the case of 3'AMP could well be due to the interaction of the adenine moiety in the B_2 site which, due to the postulated effect of weakening the interaction with Asp-121, would cause a further decrease of the pK_2 value of His-119. The similarity between derivative II and 3'AMP suggests that 3'AMP binds preferentially in $B_2R_2p_2$. This hypothesis could explain the upfield shift observed in the titration curves of His-119 in the presence of 3'AMP as due to a stacking interaction between the two heterocyclic rings. In this case, the shift could not be masked by the interaction of the phosphate in p_1 as in the case of 5'AMP.

b. 5'AMP, 5'GMP and 5'IMP. The pK values found on interaction of the enzyme with 5'AMP are in good agreement with the data already published (Table II) [6,8]. An examination of the pK values shows that while for 5'AMP a low His-12 and a high His-119 pK_2 value are measured (6.52 and 7.57) in the cases of 5'GMP and 5'IMP the pK trend is rather different. It is generally accepted that 5'AMP binds mostly to $B_2R_2p_1$ [1,6,9]; the low value for the pK_2 of His-12 (6.52) is explained by the lack of strong interaction in the B_1 site while the high pK_2 value for His-119 (7.57) confirms the binding of the phosphate in the p_1 site.

The rather different values for 5'GMP and 5'IMP cannot be interpreted on the assumption of a single preferential binding site. It is suggested that the intermediate values for the pK_2 of both histidine residues reflect a competition for more than one phosphate-binding site. In other words, 5'GMP and 5'IMP, at the ligand/enzyme molar ratio of 6 : 1, can bind not only in $B_2R_2p_1$ but also in $B_1R_1p_0$. This hypothesis would explain the intermediate pK_2 value for His-12 (7.51 and 7.11) and since the His-12 pK_2 value can be considered as a probe for the interaction in B_1R_1, the higher value for His-12 in the case of 5'GMP would indicate a higher affinity for B_1R_1 than that of 5'IMP and 5'AMP. Further evidence is gained by the fact that in the presence of 5'AMP, 5'GMP and 5'IMP, there is a shift of an aromatic resonance which infers an interaction in B_1 and that X-ray studies postulate that 3'AMP and adenosine 3',5'-phosphate appear to bind weakly in B_1 [1]. In addition, the fact that in the hydrolysis of poly A [43] a cyclic intermediate is obtained, also suggests that there is an interaction of the adenosine part of poly A with B_1R_1.

c. cl^{6}Pur-RibP and br^{8}AMP. The two substituted nucleotides cl^{6}Pur-RibP and br^{8}AMP show different behaviours. The interpretation of the br^{8}AMP is rather straightforward on the basis of the His-12 and and 119 pK_2 values. The low value of the former and the high value of the latter indicate that this analogue is mostly bound to $B_2R_2p_1$. It is worth pointing out that br^{8}AMP has a *syn* structure [44–47] as in the case of 8-oxoadenosine 3'phosphate [28] for which an interaction in $B_1R_1p_1$ has been postulated [8,29]. It is suggested that in the case of 8-oxoadenosine 3'-phosphate the pK values for His-12 and 119 found by Haar et al. [8] and

Antonov et al. [29], on the basis of the considerations already used for the other purine nucleotides could be interpreted in terms of interaction in both $B_1R_1p_1$ and $B_2R_2p_2$.

In the case of cl^6Pur-RibP the low increase in the pK_2 value of His-12 is in agreement with the lack of aromatic resonance shift, that is to say, no interaction in $B_1R_1p_0$. The low pK_2 for His-119 would partially reflect the lower association constant observed between nucleotide and enzyme with respect to other natural and halogenated nucleotides [14]. Nevertheless, the clear increase of the pK_2 values for both histidine residues with respect to the native enzyme or to derivative II, indicates different positions of the phosphate in the covalent derivative II and in the interaction of the halogenated mononucleotide with RNAase A as postulated [10]. The nitrogenous base of cl^6Pur-RibP interacts in B_2 as shown by the upfield shift present along the whole titration curve of His-119 (except around pH 6.5) probably indicating, as in the case of 3′AMP, a stacking interaction between the purine and imidazole rings.

In conclusion, the results obtained in this work show that, in derivative II, the phosphate of the labelling nucleotide is located in a position different from that occupied by the phosphate when a free 5′ purine mononucleotide interacts with native RNAase A. The perturbation of the cationic cluster around His-12 and 119, together with other data from the literature, suggest that Lys-7 and/or Arg-10 may be part of the phosphate-binding site p_2 postulated by Parés et al. [10].

The pK values of His-12 and 119, the chemical shift data and the upfield shift of an aromatic resonance on the interaction of some mononucleotide 5′-monophosphates with the native enzyme show that the natural purine nucleotides tested interact not only in $B_2R_2p_1$ but also in $B_1R_1p_0$. The strength of this latter interaction would follow the order 5′GMP > 5′IMP > 5′AMP.

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