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Evidence on the Existence of a Purine Ligand Induced Conformational Change in the Active Site of Bovine Pancreatic Ribonuclease A Studied by Proton Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: The titration curves of the C-2 histidine protons of RNase A and of derivative II—a covalent derivative obtained by reaction of the enzyme with the halogenated nucleotide 9-β-D-ribofuranosyl-6-chloropurine 5′-phosphate—in the presence of a number of purine nucleosides, nucleoside monophosphates, and nucleoside diphosphates were studied by means of proton nuclear magnetic resonance at 270 MHz. The examination of the perturbations found on the chemical shifts and p*K*s of the C-2 protons of His-12, -48, and -119 are consistent with the following conclusions: (1) The interaction of adenosine in the primary purine binding site of the enzyme (B_2R_2) induces a conformational change in the active center of the enzyme [for the nomenclature of the RNase A binding subsites, see Parés et al. [Parés, X., Llorens, R., Arûs, C., & Cuchillo, C. M. (1980) *Eur. J. Biochem. 105*, 571–579]]. (2) The phosphate moiety of the ligands, independently of its position, probably acts as a general carrier of the nucleotide to the active center, while the substituents of the base are the generators of the specificity of the binding and control the binding equilibrium between subsites B_2R_2 and B_1R_1. (3) There is no overlapping between the binding sites occupied by the labeling nucleotide in derivative II ($B_3R_3p_2$) and the primary binding site for purine mononucleotides ($B_2R_2p_1$).

RNase A[1] hydrolyzes RNA in two steps: In the first one, the transesterification step, a cyclic phosphate is formed. This cyclic nucleotide is then hydrolyzed in the second step to the corresponding 3′-mononucleotide. The second step takes place only if the base of the cyclic nucleotide to be cleaved is a pyrimidine. However, the first step can be carried out, but at a much slower rate, even if the base in the 3′ position is a purine (Richards & Wyckoff, 1971; Cozzone & Jardetzky, 1977). The rate of hydrolysis in the first step is faster when a purine nucleoside is attached to the 5′ position, reaching its highest value with adenosine (Witzel & Barnard, 1962). Free adenosine also increases the rate of the second step of the catalysis (Wieker & Witzel, 1967). The studies carried out to clarify the mechanism by which this enhancement is produced have given general information about the binding subsites of RNase A. X-ray diffraction studies (Richards &

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; Ado, adenosine; Guo, guanosine; N^1-oxoAdo, N^1-oxoadenosine; cl^{6}PurRib, 9-β-D-ribofuranosyl-6-chloropurine; cl^{6}RMP, 9-β-D-ribofuranosyl-6-chloropurine 5′-phosphate; br^{8}Ado, 8-bromoadenosine; Up[CH_2]A, 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 $-CH_2$ group; C2′–5′A, cytidylyl(2′–5′)adenosine; CM, carboxymethyl; NMR, nuclear magnetic resonance. The nomenclature of the RNase A binding subsites is found in Parés et al. (1980). B stands for base, R for ribose, and p for phosphate. $B_1R_1p_1$ is the main 3′-pyrimidine nucleotide binding site and $B_2R_2p_1$ the main 5′-purine nucleotide binding site. p_0 and p_2 are adjacent phosphate-binding sites.

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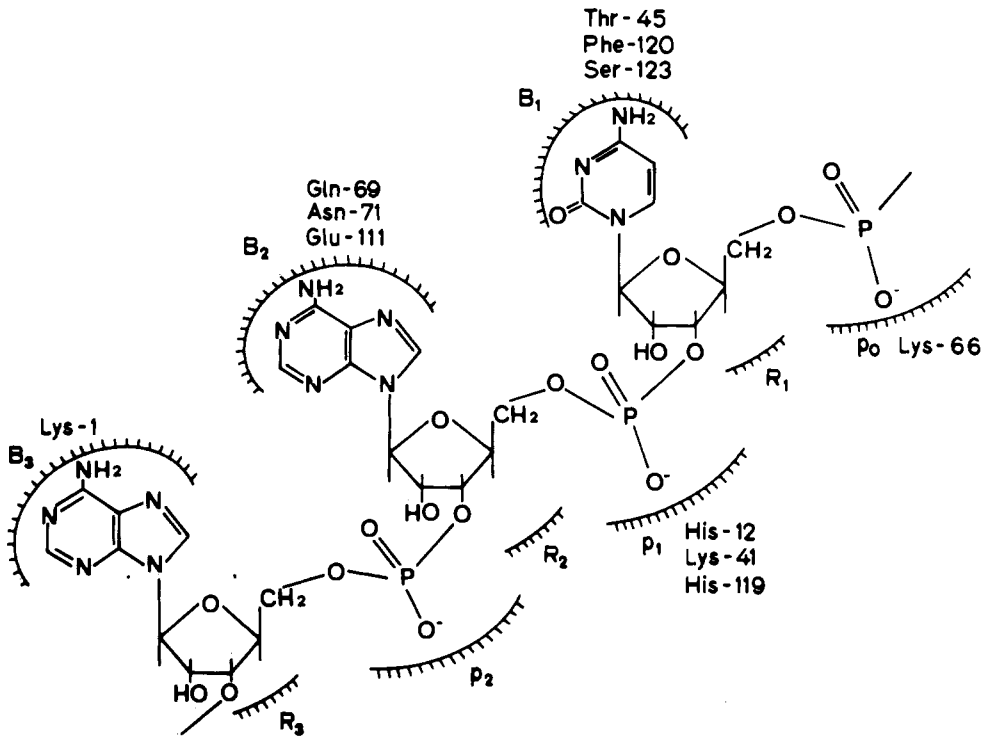

FIGURE 1: Schematic illustration of binding of an RNA fragment to RNase A. In each subsite are listed the amino acids the participation of which is known or postulated. $B_1R_1p_1R_2B_2$ is the region occupied by the substrate analogues Up[CH_2]A (Richards & Wyckoff, 1971) and C2′–5′A (Wodak et al., 1977). The cleavage takes place at the P–O-5′ bond of the phosphate occupying p_1. 5′-AMP binds preferentially to $B_2R_2p_1$ and 3′-CMP to $B_1R_1p_1$ (Richards & Wyckoff, 1971). $B_3R_3p_2$ would be the site occupied by cl^{6}RMP when it reacts with Lys-1 to yield derivative II (Parés et al., 1980).

Wyckoff, 1971) established preferential binding subsites for purine and pyrimidine nucleotides ($B_2R_2p_1$ and $B_1R_1p_1$, respectively) (see Figure 1). Other X-ray diffraction (Mitsui et al., 1978) and affinity labeling (Parés et al., 1980) studies have shown the existence of two additional phosphate binding subsites, p_0 and p_2, respectively.

Some NMR studies (Meadows et al., 1969; Haffner & Wang, 1973; Griffin et al., 1973; Haar et al., 1974; Antonov et al., 1978) have given general information about the interaction of different ligands with the enzyme, and recent work in this lab (Arús et al., 1981) has shown the existence of an equilibrium of the purine nucleotides between $B_2R_2p_1$ and $B_1R_1p_0$. All these data make it clear that in order to draw any conclusions about the interaction of ligands with the enzyme, it is necessary to take into account the influence of the substituents of the base and the position of the phosphate in this equilibrium between different binding subsites.

According to the previous considerations the main goal of the present work was to characterize the effect produced by the interaction of purine ligands in the B_2R_2 binding subsite of the enzyme. To this end, the role of various substitutions on the base and the effect of both position and number of phosphate groups in the interaction with the different subsites were studied. For carrying out that work, profit was taken of the sensitivity of the pK values and of the chemical shifts of the C-2 imidazole protons of three out of the four histidines of RNase A on interaction with different ligands.

Materials and Methods

RNase (twice crystallized) was from Cambrian Chemicals (Croydon, Surrey, U.K.). This material was purified by the method of Taborsky (1959) to obtain the RNase A fraction. The method was modified in that CM-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) was used instead of the original CM-cellulose. 5′-AMP·Na_2, adenosine, and guanosine were from Merck (Darmstadt, German Federal Republic). 3′-AMP and ribosyl-6-chloropurine were from Cambrian Chemicals. 5′-ADP·NaK was from Boehringer (Mannheim, German Federal Republic). 8-Bromoadenosine was from Calbiochem (Los Angeles, CA). 3′-GMP, N^1-oxoadenosine, and 3′,5′-ADP were from Sigma (St. Louis, MO).

C^2H_3COONa was obtained by reaction of equimolar amounts of $C^2H_3COO^2H$ (Aldrich, Milwaukee, WI) and NaO^2H (Aldrich), followed by repeated freeze-drying from ^2H_2O (99.98% isotopical purity) (Aldrich). Derivative II, a covalent derivative obtained by reaction of RNase A with cl^{6}RMP, was obtained according to Parés et al. (1980).

The exchangeable protons of the protein samples were deuterated by following the procedure of Patel et al. (1975a). Final solutions of RNase A and derivative II (60 mg·mL^{-1}) were made in ^2H_2O, 0.2 M in C^2H_3COONa, to which the desired amount of nucleotide was added. The [ligand]/[enzyme] molar ratio in the titration experiments was always 6/1, except for Guo and br^{8}Ado in which the ratio was 1/1 due to solubility problems. The pH was measured in the NMR sample tube with a special combined electrode (Microelectrodes Inc., Londonderry, NH) 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, before and after recording the spectra, were direct meter readings. They were accepted only if they agreed to within 0.04 pH unit.

A 270-MHz Brucker 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. Chemical shifts are reported in parts per million downfield from internal $(CH_3)_3Si(CH_2)_3SO_3Na$ (DSS) at an ambient probe temperature of 29 ± 2 °C.

Curve fitting was carried out by using a program of least-squares iterative regression fit (Von Meerwall, 1976),

Table I: Apparent pK Values of Histidine and Other Residues of RNase A, Derivative II, and Enzyme–Ligand Complexes[a]

nucleotide or derivative	His-12		His-119		His-48		pK of His-105	groups influencing chemical shifts of the aromatic resonance	
	pK_1	pK_2	pK_1	pK_2	pK_1	pK_2		I	II
none	4.63 ± 1.11	6.05 ± 0.08	4.40 ± 0.01	6.32 ± 0.04	4.64 ± 1.00	6.32 ± 0.12	6.87 ± 0.04		
Ado	4.58 ± 0.45	6.10 ± 0.04	4.63 ± 0.96	6.02 ± 0.04	5.31 ± 0.26	6.30 ± 0.05	6.85 ± 0.02		5.72 ± 0.17
Ado[b]	4.98 ± 0.83	6.34 ± 0.08	5.10 ± 0.66	6.19 ± 0.05	4.23 ± 0.01	6.58 ± 0.07	6.85 ± 0.02		6.15 ± 0.26
cl^{6}PurRib	3.84 ± 0.68	5.95 ± 0.03	4.02 ± 0.22	5.89 ± 0.04	4.80 ± 0.52	6.29 ± 0.08	6.81 ± 0.02		
N^1-oxoAdo	5.05 ± 0.29	6.13 ± 0.05	4.91 ± 1.31	6.31 ± 0.05	4.52 ± 0.52	6.36 ± 0.07	6.88 ± 0.02		
Guo	4.72 ± 0.61	6.11 ± 0.04	4.47 ± 0.75	6.33 ± 0.04	4.38 ± 0.49	6.33 ± 0.06	6.83 ± 0.02		
br^8-Ado	4.53 ± 0.39	6.03 ± 0.04	4.80 ± 0.76	6.19 ± 0.06	4.58 ± 0.49	6.31 ± 0.07	6.82 ± 0.03		
5′-AMP[b]	4.77 ± 0.27	6.66 ± 0.05	4.35 ± 0.56	7.71 ± 0.03	4.88 ± 0.81	6.31 ± 0.12	6.90 ± 0.06	4.28 ± 0.03	7.77 ± 0.17
3′-AMP	4.53 ± 0.28	6.30 ± 0.04	4.96 ± 0.28	6.29 ± 0.06	5.28 ± 0.54	6.25 ± 0.16	6.86 ± 0.02		5.81 ± 0.16
3′-GMP	4.83 ± 0.07	7.62 ± 0.06	4.68 ± 0.22	6.97 ± 0.04	4.03 ± 0.51	6.69 ± 0.05	6.97 ± 0.02	4.75 ± 0.41	7.35 ± 0.15
3′,5′-ADP	4.49 ± 0.12	7.11 ± 0.03	3.63 ± 0.27	7.99 ± 0.03	4.11 ± 0.76	6.39 ± 0.07	6.98 ± 0.02	3.54 ± 0.59	7.79 ± 0.17
5′-ADP	4.00 ± 0.08	7.41 ± 0.04	3.31 ± 0.59	8.11 ± 0.04	4.82 ± 0.49	6.35 ± 0.08	6.96 ± 0.02	3.54 ± 0.07	7.40 ± 0.11

[a] When two pK values are given for a single titration curve, eq 2, assuming an imidazole–carboxyl group interaction as explained under Materials and Methods, was used. In the other cases eq 1 was used. Experiments were carried out in 0.2 M sodium acetate. [b] Experiments were carried out with derivative II; all the others were with RNase A.

adjusted for its use in a Univac 1108 computer. The following formulae (Westmoreland et al., 1975) were used: (a) For a simple proton association–dissociation equilibrium

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

where δ_{obsd} 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 case where an acid inflection was present the equation used was

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

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

It has been shown (Roberts et al., 1969) that His-12, -105, and -119 have, essentially, the same pK_a values both in NaCl and in sodium acetate. This has been confirmed later by Cohen & Shindo (1975) and Arûs et al. (1981). As a consequence, the experiments reported in this paper, carried out in 0.2 M sodium acetate solutions, in order to observe a continuous titration curve for His-48, are fully comparable with the data reported in the literature in NaCl. The existence of an acid inflection in the titration curves of His-12, -48, and -119 of RNase A, alone and in presence of different ligands, has been already reported in the literature (Rüterjans & Witzel, 1969; Schechter et al., 1972; Cohen et al., 1973; Haar et al., 1974; Cohen & Shindo, 1975; Markley & Finkenstadt, 1975; Antonov et al., 1978). According to that, and as it is possible to see in Figures 2, 4, and 5 and Table I, the existence of an acid inflection was taken into account in most cases in order to obtain a more accurate pK_2 value. The large error of pK_1 values is due, in all cases, to the uncertainty in the experimental points (±0.02 ppm), which is close to the width of the acid inflection (Δ_1). This fact does not allow any useful comparative studies of their values, except for two cases in which Δ_1 is big enough to allow an accurate determination of the pK_1 (see 3′,5′-ADP, Figure 5c with Δ_1 for His-12 = 0.34 ppm and Δ_1 for His-119 = 0.30 ppm, and 5′-ADP, Figure 5d with Δ_1 for His-12 = 0.46 ppm and Δ_1 for His-119 = 0.16 ppm). The origin of these acid inflections was proposed to be the interaction, either directly or through a conformational change, of one or more carboxylate groups with His-12, -48, and -119 (Cohen & Shindo, 1975). While the interactions of Asp-121 with His-119 (Richards & Wyckoff, 1971; Wlodawer & Sjölin, 1981) and Asp-14 with His-48 (Santoro et al., 1979; Walters & Allerhand, 1980) are widely accepted, and could then be the cause of the observed acid inflections, the interaction, through conformational change, between Asp-14 and His-12 seems doubtful (Niu et al., 1979).

All perturbations found on interaction of a ligand with the enzyme have been attributed to the different binding subsites according to the established criteria (Arûs et al., 1981). The alteration of a given binding subsite probe due to the interaction of the ligand in another subsite (induced fit) has not been considered.

Interaction of Purine Nucleosides with RNase A and with Derivative II. (A) Adenosine, Ribosyl-6-chloropurine and N^1-Oxoadenosine. A clear difference can be established between the former two ligands and N^1-oxoAdo. The latter does not perturb either the chemical shift or the pKs of the four histidines (Table I). In contrast, both Ado and cl^{6}PurRib perturb His-119 when they interact with RNase A, producing a high-field shift (0.1–0.2 ppm), more pronounced in the case of cl^{6}PurRib than in the case of Ado, especially at neutral and basic pH, and a decrease of its pK (0.3–0.4 pH unit). Similar effects are observed in the case of derivative II plus Ado (Figure 2, Table I), indicating that occupation of $B_3R_3p_2$ by the labeling nucleotide in derivative II (Parés et al., 1980; Arûs et al., 1981) does not interfere with the interaction of the nucleoside ligand in B_2R_2. The slight difference existing between the pK_2 of the active center histidines of derivative II and RNase A (Arûs et al., 1981) is maintained after interaction with Ado (Table I). This also suggests that the interaction of Ado in B_2R_2 is independent of the occupation of $B_3R_3p_2$ by the labeling nucleotide. The observed high-field shift in the titration curves of the C-2 proton of His-119 in the case of cl^{6}PurRib plus RNase A does perfectly agree in quality and magnitude with the effect observed on the interaction of RNase A with cl^{6}RMP (Arûs et al., 1981), indicating a similar interaction of the nucleoside moiety in both cases. The strong decrease of the pK_2 value of His-119 (~0.4 pH unit) would also agree with the lower pK_2 value of His-119 in the case of cl^{6}RMP as compared with other purine nucleotides (6.8 vs. 7.1–7.6; Arûs et al., 1981).

The high-field shift of the titration curve of His-119 in these two cases (Ado and cl^{6}PurRib) could be due to a stacking between the heterocyclic rings of the histidine and the nu-

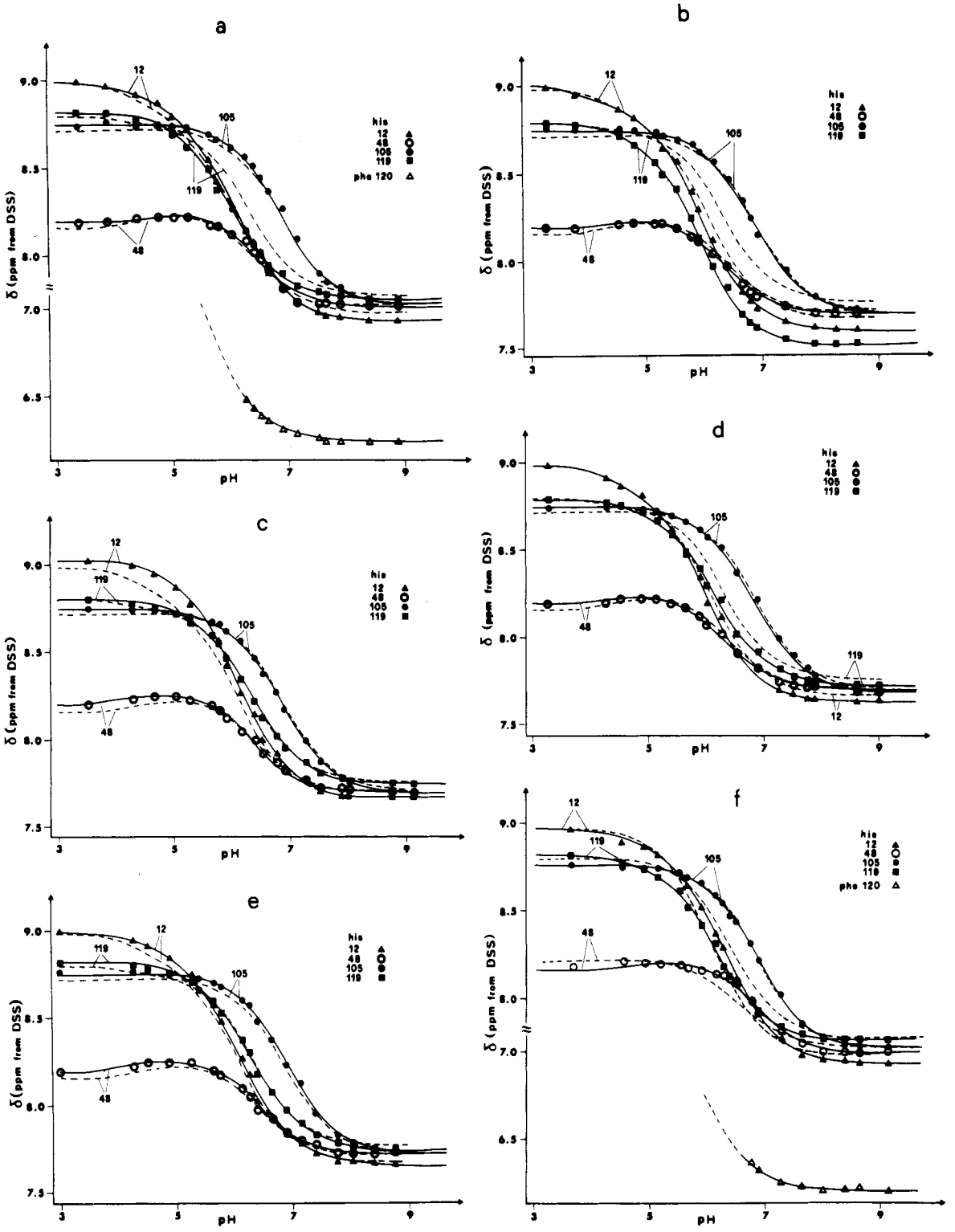

FIGURE 2: pH dependence of chemical shifts of histidine C-2 proton resonances of RNase A. The curves were obtained in the presence of (a) adenosine, (b) ribosyl-6-chloropurine, (c) guanosine, (d) 8-bromoadenosine, and (e) N^1-oxoadenosine. Panel f shows the titration of the histidines of derivative II in the presence of adenosine. 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 RNase A. The conditions used were 4.4 mM RNase A (or derivative II) in 0.2 M sodium acetate. The [ligand]/[enzyme] molar ratio was 6/1 in all cases except for Guo and br^{8}Ado in which it was 1/1.

cleoside, stabilized by some transfer of charge density. It is known that purines and imidazoles can form weak charge–transfer complexes (Slifkin, 1971) in which the histidine in the neutral form would act as a donor and the purine as an acceptor. The existence of such a complex is known to cause perturbation in the chemical shifts, their sign depending on the interacting compounds (Slifkin, 1971).

Nevertheless, the existence of this effect would not explain the decrease of the pK_2 of His-119. Therefore such a decrease must be caused by some charge perturbation of the environment of His-119 on interaction of the nucleosides with the enzyme. It is postulated that, as advanced in a previous paper (Arûs et al., 1981), the interaction in B_2R_2 of a purine ring with a particular structure (N^1-oxoAdo would not) positions His-119 and Asp-121 in such a way that the interaction existing in the free enzyme is weakened, and as a result the pK_2

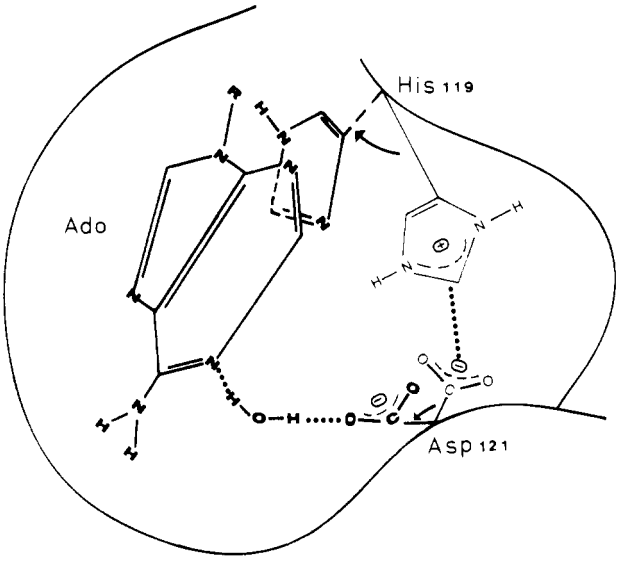

FIGURE 3: Schematic illustration of proposed conformational change induced by adenosine with RNase A. The enzyme residues in the free form are represented in lighter drawing than the same residues after interaction with adenosine. The interaction between Asp-121 and His-119 (electrostatic and/or hydrogen bonding) would be broken when Ado occupies B_2R_2. Asp-121 would then interact, directly or through a water molecule, with the N^1 position of the purine ring. His-119 would also move to a more favorable position for a stacking with the heterocyclic ring occupying B_2.

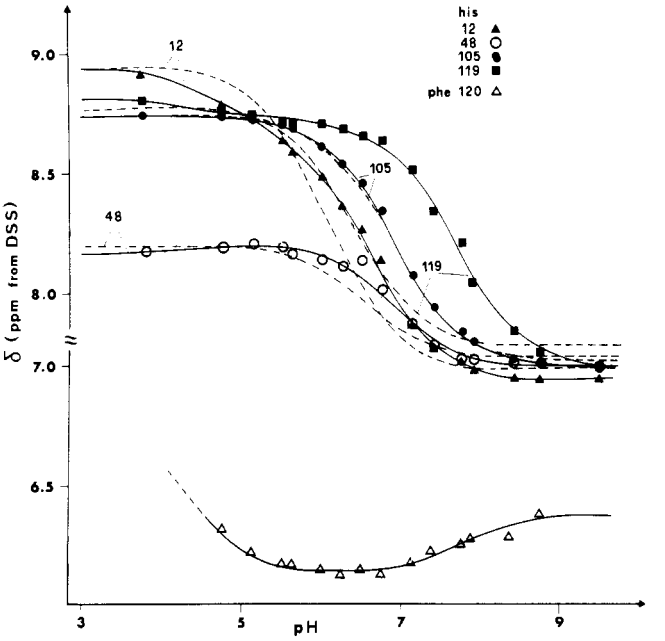

FIGURE 4: pH dependence of chemical shifts of histidine C-2 proton resonances of derivative II plus 5′-AMP. For comparison the histidine titration curves of free derivative II are also included (dashed lines). Numbers refer to the histidine residues in the sequence of RNase A. The conditions used were 4.4 mM derivative II in 0.2 M sodium acetate. The [ligand]/[enzyme] molar ratio was 6/1.

of His-119 decreases. This would increase the amount of histidine in the neutral form and thus favor the charge–transfer to the purine, which might cause the observed downfield chemical shift.

The existence of hydrogen bonding between Asp-121 and the heterocyclic ring of Ado, through a water molecule, has already been postulated by the theoretical studies of Deakyne & Allen (1979). It is also known that the N^1 position of purines can be a place of direct (no water involved) hydrogen bonding between carboxylic acids and Ado in nonpolar media (Lancelot, 1977; Gresh & Pullman, 1980). Moreover, the UV difference spectrum produced by the interaction of 5′-AMP and RNase A can be explained (Deavin et al., 1968; Parés et al., 1978) by a combination of a protonation in N^1 or N^7 and some increase of the hydrophobicity of the environment of the heterocyclic ring. All these facts would agree with the complexation of Ado and RNase A affecting the equilibrium of the interaction of Asp-121–His-119 as indicated in the scheme of Figure 3. The interaction of N^1-oxoAdo with the enzyme would not cause the same effect as Ado or cl^{6}PurRib due to the substitution in the crucial N^1 position, which would affect the orientation of the nucleoside in the B_2R_2 site and its interaction with Asp-121 and His-119.

No effect was observed on the titration curves of the other three histidines, except a slight increase of the pK_2 of His-12 in the case of derivative II plus Ado (~0.2 pH unit). This could indicate a stronger interaction of Ado in B_1R_1 when $B_3R_3p_2$ is occupied as is the case in derivative II. It is well-known (Markus et al., 1968; Gregory et al., 1978) that the three-dimensional structure of RNase A is more compact in the presence of nucleotides. This fact should affect the interaction of the enzyme with the ligand in the other binding sites, although other explanations are also possible.

Only in the case of Ado, both with RNase A and with derivative II, was it possible to observe the high-field shift of an aromatic resonance assigned, as in the case of other pyrimidine and purine nucleotides, to two of the protons of the Phe-120 ring (Santoro et al., 1979; Lenstra et al., 1977; Arús et al., 1981). This fact would indicate that not only 5′-AMP but also its corresponding nucleoside are in equilibrium between B_2R_2 and B_1R_1 as shown for some 5′-purine nucleotides (Arús et al., 1981). Only a single pH inflection in the zone around pH 6 could be observed for this aromatic perturbation, suggesting that the nonprotonated form of a group with such a pK value is responsible for the interaction of Ado in B_1R_1, a likely candidate for this role being His-119. In order to understand how His-119 could affect the interaction of Ado in B_1R_1, it is postulated the existence of two different equilibria between free ligand and ligand bound in the primary binding site (B_2R_2) on one hand and ligand bound in the primary binding site and ligand bound in the secondary binding site (i.e., B_1R_1) on the other. This latter equilibrium on the surface of the enzyme would also explain why two pH inflections very similar in value to the pK_1 and pK_2 of His-119 are shown by the aromatic resonance following the interaction of 5′-AMP, 5′-GMP, and 5′-IMP with RNase A in the $B_1R_1p_0$ site (Arús et al., 1981).

(*B*) *Guanosine and 8-Bromoadenosine.* The low [ligand]/[enzyme] molar ratio used (1/1) may explain the lack of appreciable effect on the p*Ks* of the active center histidines. Nevertheless, the chemical shift data of the titration curves are, in these cases, a more sensitive probe for the interaction of the ligand. In the interaction of Guo with the enzyme, it is possible to observe a slight, but reproducible, downfield shift (0.07 ppm) in the acid zone of the titration curve of His-12, showing that in this case there is also an interaction in B_1R_1, probably in equilibrium with B_2R_2 as in the case of 5′-GMP (Arús et al., 1981).

In the case of br^{8}Ado only a slight high-field shift in the titration curve of His-119 could be monitored in agreement with the above experiments with Ado and cl^{6}PurRib. However, in the presence of br^{8}Ado this shift is not reflected in a clear pK_2 decrease of His-119.

Interaction of 5′-AMP with Derivative II. The p*K*s of His-12 and His-119 of derivative II in the presence of 5′-AMP and the shape of their titration curves (Table I and Figure 4)

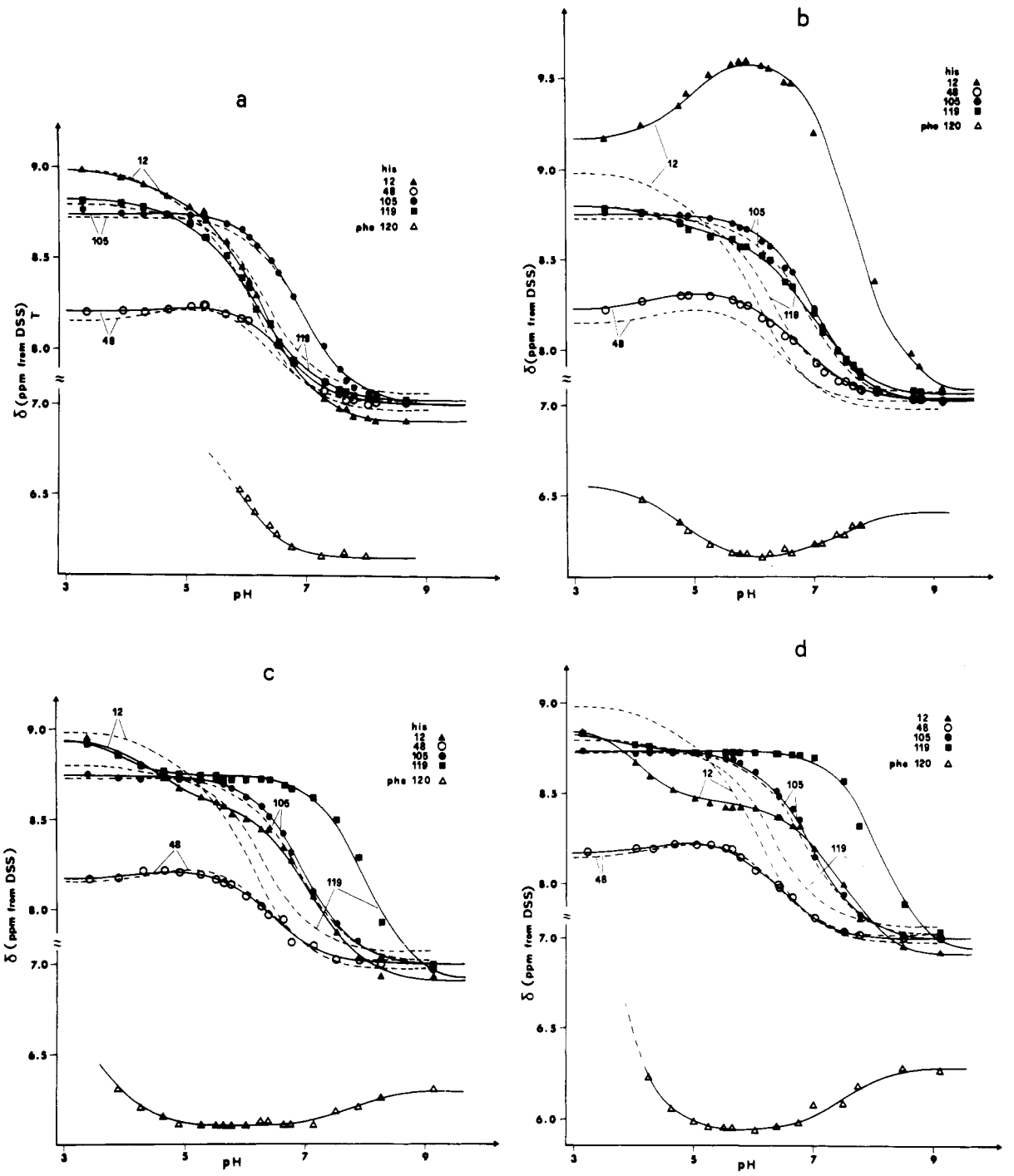

FIGURE 5: pH dependence of chemical shifts of histidine C-2 proton resonances of RNase A. The experiments were carried out in the presence of (a) 3′-AMP, (b) 3′-GMP, (c) 3′,5′-ADP, and (d) 5′-ADP. For comparison the histidine titration curves of the free enzyme are also shown (dashed lines). Numbers refer to the histidine residues in the sequence of RNase A. The conditions used were 4.4 mM RNase A in 0.2 M sodium acetate. The [ligand]/[enzyme] molar ratio was 6/1 in all cases.

agree with the previously published values for the interaction of 5′-AMP with RNase A (Haffner & Wang, 1973; Haar et al., 1974; Arûs et al., 1981). These results show that the accessibility of the purine nucleotides to $B_2R_2p_1$ in the derivative II is the same as in RNase A. The perturbation of the chemical shift of the aromatic resonance assigned to Phe-120 and the rather low pK_2 of His-12, compared with other purine nucleotides, indicate the existence of a weak interaction of 5′-AMP in B_1R_1 as in the case of RNase A (Arûs et al., 1981).

Interaction of 3′-Mononucleotides with RNase A. 3′-AMP interacts weakly with the enzyme as compared with 5′-AMP, even at a [ligand]/[enzyme] molar ratio of 6/1. The pK_2 values of the active center histidines (Table I) are in agreement with previously reported values (Haar et al., 1974) obtained at a lower [ligand]/[enzyme] molar ratio (1.8/1). The slight increase of the pK_2 of His-12 indicates an interaction in $B_1R_1p_1$, while the absence of perturbation of the pK_2 of His-119 should be interpreted on the basis of the results obtained with Ado. In other words, two opposite effects contribute to the final value of the pK_2 of His-119 in the 3′-AMP case: the purine nucleoside occupying B_2R_2 lowers the pK_2 while the phosphate in p_1 ($B_1R_1p_1$) or in p_2 ($B_2R_2p_2$) increases it. Apparently, in the case of 3′-AMP both effects cancel each other at a molar ratio of 6:1.

The case of 3′-GMP is quite different. The perturbation

of the titration curve of His-12 (Figure 5) is very similar to that produced by interaction of 5′-GMP with RNase A (Arûs et al., 1981). There is a large downfield shift all along its titration curve even at acid pH values, an extensive broadening of the resonance at neutral pH, and a high value (7.62) of its pK_2 (Table I). All these facts demonstrate the strong interaction of 3′-GMP in $B_1R_1p_1$. These data show the importance of the nucleoside structure, more than the position of the phosphate in the ribose ring, in determining the final equilibrium of the ligand between the different binding sites of the enzyme. The lower pK_2 of His-119 with respect to the values obtained with 5′-GMP (6.97 vs. 7.57) could be caused, as in the case of 3′-AMP, by a different orientation of the phosphate in p_1 when coming from a purine nucleoside occupying B_1R_1 or B_2R_2, by an interaction of the ribose 5′-O with His-119 (Deakyne & Allen, 1979), absent when the ribose is in R_1, or by both.

His-48 is clearly perturbed on interaction of 3′-GMP with RNase A. As it is also seen with pyrimidine nucleotides (Rüterjans & Witzel, 1969; Haar et al., 1974) 5′-GMP and 5′-IMP (Arûs et al., 1981), there is a downfield shift (0.1 ppm) of its titration curves at acid pH, indicating an interaction in B_1R_1 (Arûs et al., 1981). In the case of 3′-GMP there is also a clear increase of the pK_2 of His-48 (0.35 pH unit). Unfortunately there are no data available on the perturbation of the pK_2 of His-48 by pyrimidine nucleotides due to the disappearance of the His-48 signal in NaCl solutions, conditions used in the bibliography to follow the interaction, at neutral pH (Meadows & Jardetzky, 1968; Roberts et al., 1969). As a consequence it is difficult to decide whether or not the increase of the pK_2 of His-48 in the case of 3′-GMP can be taken as a proof of a B_1R_1 interaction stronger than in the case of 5′-GMP in which no change in the His-48 pK_2 could be monitored (Arûs et al., 1981), affecting through a conformational change the triad His-48–Asp-14–Tyr-25 (Markley, 1975; Cohen & Shindo, 1975; Santoro et al., 1979; Lenstra et al., 1977).

Both 3′-AMP and 3′-GMP produce a high-field shift of the aromatic resonance assigned to Phe-120 (Figure 5), indicating an interaction in B_1R_1. In the case of 3′-AMP the pH inflection is similar to that observed in the interaction of Ado with both RNase A and derivative II, and only one pH inflection around neutrality with a pK similar to one of the active center histidines could be found. However, in the case of 3′-GMP the resonance shows two pH inflections as in the case of 5′-GMP.

Interaction of Nucleoside Diphosphates with RNase A. (A) 3′,5′-ADP. The slight increase (0.3–0.4 pH unit) of the pK_2 of His-119 with respect to that found with 5′-AMP is in agreement with the interaction of the 3′-phosphate in the p_2 binding site in a way similar to that of the phosphate of the labeling nucleotide in derivative II. Nevertheless, there is a clear increase (1.07 pH units) of the pK of His-12 with respect to native RNase A, which is 0.6–0.7 pH unit more than the effect that could be expected from the presence of a second phosphate in p_2. This increase has to be explained by a stronger interaction of 3′,5′-ADP in $p_1B_1R_1p_0$ than that of 5′-AMP in $B_1R_1p_0$. In the case of 3′,5′-ADP it is safe to say that the origin of the acid inflections for His-12 and His-119 must be different because, even taking into account the uncertainties in the computed values, the pK_1 for His-119 is clearly lower than that for His-12 (Table I).

(B) 5′-ADP. The pK data (Table I) indicate that the interaction of 5′-ADP with RNase A is very strong and that both phosphates can be accommodated in p_1. The high pK for both histidines (12 and 119) can be interpreted in terms of the strong destabilization of the cationic cluster around His-12 and His-119 (Richards & Wyckoff, 1971; Patel et al., 1975b) by the presence of two vicinal phosphates. In this case, as with 3′,5′-ADP, the pK_1 values for His-12 and His-119 indicate a different origin for both acid inflections. Both 3′,5′-ADP and 5′-ADP cause a high-field shift of the aromatic resonance assigned to Phe-120 (Figure 5), also in agreement with their interaction in B_1R_1.

In conclusion, the data presented in this paper point to the existence of a ligand-induced conformational change, in which Asp-121 and His-119 would be involved, on interaction of RNase A with certain purine nucleosides. The existence of this conformational change induced by Ado, a molecule known to enhance the catalytic activity of the enzyme both in the first and in the second step of the catalysis, and its absence with N^1-oxoAdo, which does not activate either the first (Follman et al., 1967) or the second step of the catalysis (Wieker & Witzel, 1967), would point to this conformational change as an important factor in the activation of the hydrolysis of dinucleoside monophosphates when a purine nucleoside occupies the B_2R_2 position.

The data presented here show that the function of the phosphate is more that of a general carrier of the ligand to the target (the active center of the enzyme) than as a generator of specificity of binding. The experiments of 3′-GMP and Guo when compared to those of 5′-GMP clearly show the importance of the nucleoside structure in the equilibrium of the ligand between the binding subsites B_2R_2 and B_1R_1.

It is also clear from the experiments carried out with derivative II that there is no overlapping between the $B_2R_2p_1$ and $B_3R_3p_2$ binding sites (Parês et al., 1980), the latter one being occupied by the labeling nucleotide in the covalent derivative of the enzyme. An analogous derivative should be used to know whether or not there is overlapping between $B_2R_2p_1$ and $B_1R_1p_0$.

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Identification and Partial Purification of a Factor That Stimulates Calcium-Dependent Proteases†

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ABSTRACT: We have identified and partially purified from bovine brain a factor which stimulates the activities of two rat liver calcium-dependent proteases. The factor, which is physically and functionally distinct from calmodulin, is heat stable and has a low apparent molecular weight (M_r 20000). The factor stimulates rates of proteolysis up to 25-fold but does not alter the calcium sensitivity of either protease. We have called this factor calcium-dependent protease regulator.

Recently we have been studying two rat liver cytoplasmic proteases which are completely dependent on calcium for activity. These enzymes are distinguishable by different calcium concentration requirements (DeMartino, 1981). Because the activities of many calcium-dependent enzymes are regulated by the calcium-binding protein calmodulin (Klee et al., 1980; Cheung, 1980), we examined the effects of calmodulin on the calcium-dependent proteases. In our initial experiments (DeMartino & Kuers, 1981) both proteases were greatly stimulated by samples of bovine brain calmodulin which had been prepared in a manner similar to previously published methods (Dedman et al., 1977). However, in subsequent experiments, neither protease was affected by highly purified bovine brain calmodulin which had been prepared by affinity chromatography on fluphenazine–Sepharose. Despite the different protease stimulatory activities of the two calmodulin preparations, each was able to activate the calmodulin-dependent enzyme, myosin light chain kinase. These results suggested that the factor responsible for the protease stimulatory activity in the original preparation was distinct from calmodulin. The purpose of the present work was to determine whether this possibility was correct and, if so, to identify and to characterize this stimulatory activity.

Materials and Methods

Preparation of Rat Liver Calcium-Dependent Proteases. Calcium-dependent proteases from rat liver were prepared as described previously (DeMartino, 1981). After isolation by DE52 ion-exchange column chromatography, each protease was dialyzed against 50 mM Tris-HCl,[1] pH 7.5, 0.5 mM

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; DTT, dithiothreitol; Mops, 3-(*N*-morpholino)propanesulfonic acid; CDPR, calcium-dependent protease regulator; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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