NMR Studies on Interactions of Ribonuclease Sa with Guo-3′-P

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Abstract. Some features of the interaction of guanyloribonuclease Sa from *Streptomyces aureofaciens* with its competitive inhibitor Guo-3′-P were investigated by ^{1}H and ^{31}P NMR spectroscopy. The pH dependence of chemical shifts of C(2)-H protons of the histidine residue of the enzyme were analysed, in the absence and presence of Guo-3′-P. This analysis showed that only one of the two histidines of ribonuclease Sa is located in the active site of the enzyme. ^{31}P NMR resonances of the nucleotide and of its complex with the enzyme indicated that this histidine interacts with the phosphate group of the substrate. The possible relationship between the observed perturbation of the NMR titration curve of the active site of histidine and a conformational change in the enzyme molecule at a pH of approximately 7.5 is also discussed.

Key words: Guanyloribonuclease Sa — NMR spectroscopy — Guo-3′-P

Introduction

Due to their narrow specificity, good stability and low molecular weight, specific ribonucleases, especially guanyloribonucleases from various sources, have been recently shown to be suitable material for comparative studies of molecular basis of the specific actions of enzyme. Ribonuclease T_1 from *Aspergillus oryzae* has been extensively studied and is well known. Detailed kinetic and spectroscopic studies of the active sites of many other guanyloribonucleases have also been carried out (Bezborodova and Bezborodov 1979).

Ribonuclease Sa (E.C.3.1.4.8.) isolated from *Streptomyces aureofaciens* is a guanylate specific endoribonuclease. This enzyme catalyses the splitting of RNA only at sites where a guanosine nucleotide residue is located in the 3′-position of 3′—5′-phosphodiester linkages along the RNA chain (Zelinková et al. 1971). Its amino acid composition (Ševčík et al. 1982) differs considerably from that of ribonuclease T_1. Our preliminary NMR measurements indicated that at least 1 histidine residue is located in or near the ribonuclease Sa active site (Rüterjans et

al. 1976). Chemical modification experiments revealed that the active site does not contain arginine or lysine residues which could bind the negatively charged phospate group of the substrate. Kinetic measurements indicated that this binding function could belong to a histidine residue. An indication for the engagement of tyrosine residues in the enzyme — substrate interaction has been also obtained (Both et al. 1982).

NMR spectroscopy has proved to be a powerful tool for the elucidation of submolecular interactions with enzymes. Up to now changes in chemical shifts of the imidazole ring C(2)—H protons of histidine residues have generally been employed to characterize these interactions. Perturbations in the pH dependence of histidine proton chemical shifts can yield valuable information concerning the interactions of amino acid residues adjacent to the histidine residue in question (Rüterjans and Pongs 1971). Because the presence of a system of two histidine residues in the active sites of better known ribonucleases plays a decisive role in the activity of these enzymes (Bezborodova and Bezborodov 1979) it was our task to clarify the role of both histidine residues of ribonuclease Sa.

Material and Methods

Ribonuclease Sa was isolated and purified as described previously (Both et al. 1982). The enzyme used for experiments had a specific activity around 200,000 units/mg. Enzyme activity was determined according to the method of Egami et al. (1964). Lyophilised samples were dissolved in 99.8 % ^2H_2O to obtain an enzyme concentration around 1 mmol/l. The ionic strength of the solution (reached by adding NaCl) was 0.2 mol/l. In order to remove exchangeable NH-protons of the enzyme, the solution was left to stay at pH 7 and 30°C for two days. The activity of deuterium — exchanged ribonuclease Sa was not less than 90% of the original. Guo-3′-P was obtained from Sigma (U.S.A.). Other reagents were of p.a. grade.

The pH was adjusted using 0.5 mol/l solutions of ^2HCl or NaO^2H in ^2H_2O and measured using a PHM 26 pH meter (Radiometer, Denmark) equipped with special combined electrode (Ingold, F.R.G.). The pH of each sample was checked in the NMR tube (Wilmad Glass Co., U.S.A.) before and after taking each spectrum and the spectrum was accepted only if the measurements were compatible to within 0.02 pH units. The final value was used routinaly and is quoted as the uncorrected "pH meter reading". This "p^{2}H" value differs from true pH value only by about 0.1 pH unit (Roberts et al. 1969).

The NMR spectra were recorded at 30°C on a WH 270 spectrometer (Bruker, F.R.G.) equipped with a BNC 12 computer (Nicolet, U.S.A.) using the Fourier Transform mode. For stabilisation of the resonance frequencies the proton signal of residual ^1H_2O was employed. Some measurements were repeated on a XL—100—15 spectrometer (Varian, U.S.A.). On average 500 pulses were accumulated for proton spectra and around 2000 pulses for ^{31}P resonances. Chemical shifts are given in ppm from internal standard DSS (2,2,3,3,-tetradeutero-3 (trimethylsilyl)-propionic acid) for proton resonances and from external standard H_3PO_4 for ^{31}P resonances.

Results and Discussion

The aromatic region of the ^1H NMR spectrum of ribonuclease Sa at pH 5.22 is shown in Fig. 1. Ševčík et al. (1982) found 2 histidine, 6—7 tyrosine and

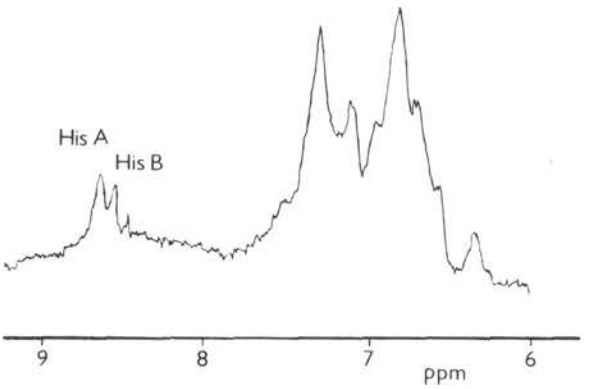

Fig. 1. Aromatic region of ^{1}H NMR spectrum of ribonuclease Sa at pH 5.22.

3 phenylalanine residues in the molecule of this enzyme. Resonance of tyrosine and phenylalanine protons and of C(4)-H protons of histidines usually occur in the region of 6—8 ppm while the resonances of histidine C(2)-H protons occur at around 8—9 ppm. In the aromatic region of ribonuclease Sa we found several resonance lines which changed their position within the pH range of 5—9. In order to identify the resonances of C(2)-H protons of histidine residues unambigously we investigated the dependence of resonance line intensities in the aromatic region of the spectra on the incubation time of the enzyme in ^2H_2O at pH 8.3 and 37°C. Under these conditions only C(2)-H histidine protons are exchanged for ^{2}H (Vaughan et al. 1970; Bradbury et al. 1973). In the investigated region of the spectra two resonance lines changed their intensity during incubation and we assigned them to the C(2)-H protons of the two histidine residues of ribonuclease Sa. In Fig. 1 these lines are designated as His A and His B. The halftimes for deuterium exchange under the above experimental conditions were 18 and 87 hours for His A and His B, respectively.

In the pH range of 6—7.5 it was very difficult to determine the positions of His A resonance lines precisely because of very wide lines which almost fully dissapeared in the noise level (Fig. 2). However, with pH difference spectra the His A resonance lines were considerably enhanced (Fig. 3) making a more exact determination of their chemical shifts possible. The spectra were recorded in the pH range of 4.7—9.0. This limited pH range was determined by the appearance of enzyme precipitation in the NMR probe cell observed outside of this pH range.

Guo-3′-P is a competitive inhibitor of ribonuclease Sa (Both et al. 1982) and it is generally assumed that its interactions with guanyloribonucleases provide a suitable model for the investigation of specific interactions between the active site

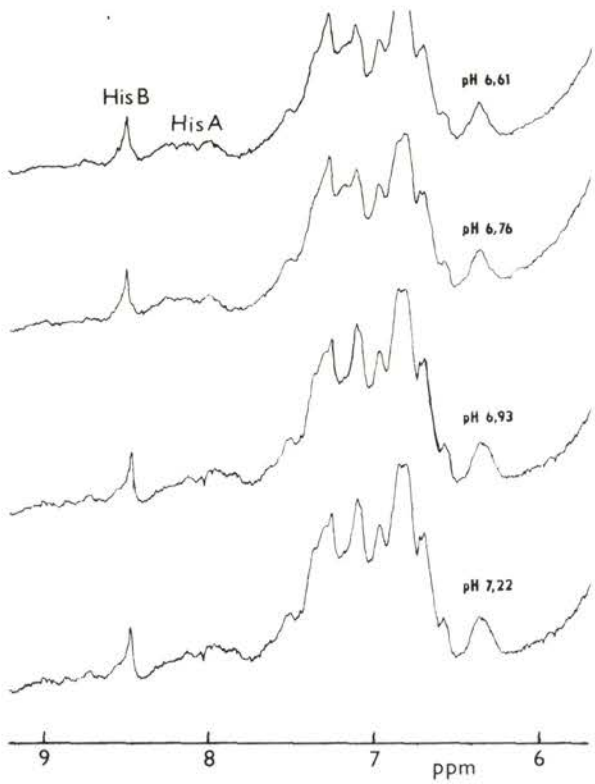

Fig. 2. ^{1}H NMR spectra of ribonuclease Sa in the pH range of 6.6—7.2.

of guanyloribonuclease and guanylic substrate. The addition of Guo-3′-P to ribonuclease Sa solution caused a marked change in the position of the His A C(2)-H resonance line and notable changes were also observed in the region of tyrosine and phenylalanine resonance lines (ribonuclease Sa does not contain tryptophane). However, the position of the His B C(2)-H resonance line was unaffected. The difference spectrum of ribonuclease Sa and its complex with Guo-3′-P at pH 5.97 and at a molar ratio of [Guo-3′-P]/[RNase Sa] = 4 is shown in Fig. 4.

By plotting the pH dependence of chemical shifts of His A and His B resonance lines of ribonuclease Sa and of its complex with Guo-3′-P titration curves of these protons were obtained as shown in Fig. 5. For the incomplete titration range (usually NMR titration is carried out over a pH range of 2—10) a precise mathematical analysis of the titration curves was not feasible and the pKa values were determined directly from the curves as their inflexion points. However, the chemical shifts for protonated states (δ_H+) and the chemical shifts for

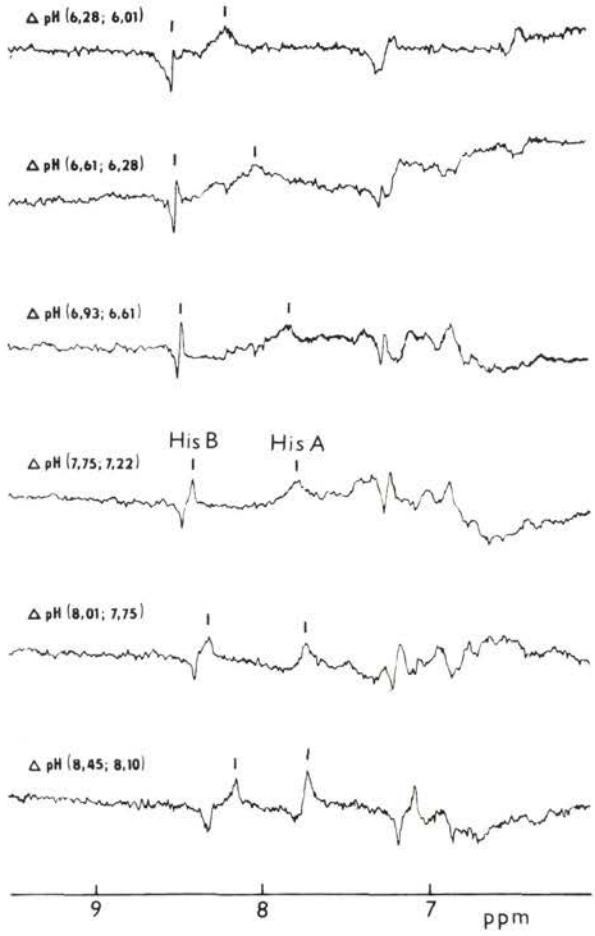

Fig. 3. ^{1}H NMR pH difference spectra of ribonuclease Sa in the pH range of 6—8.5.

deprotonated states (δ_H^0) were determined by estimation. The parameters determined by titrations are presented in Table 1.

To elucidate the character of the microenviroment of the histidine residues we compared the NMR titration parameters of His A and His B resonance lines with the titration parameters of N-acetyl-L-histidine-methylamide (AcHisNHMet) protons (Table 1). This histidine derivative simulates the case when the ionisation state of a histidine residue of the protein molecule is not affected by adjacent amino acid residues. The His A resonance line of ribonuclease Sa has very similar titration parameters to those of the C(2)-H proton of AcHisNHMet. This may indicate that

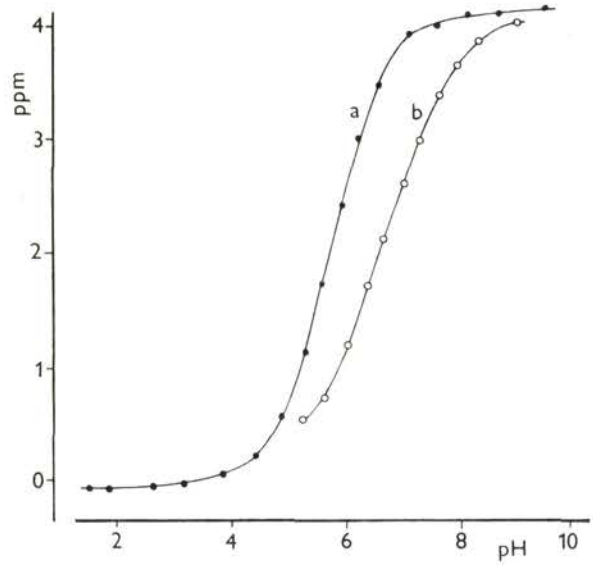

Fig. 6. pH dependence of chemical shifts of ^{31}P atom of Guo-3′-P (*a*) and its complex with ribonuclease Sa (*b*) at a molar ratio of [Guo-3′-P]/[RNase Sa] = 4.

function was observed. If our assumption concerning the relationship between the perturbation of the titration curve and the conformational change in the free enzyme is correct, than the absence of perturbation in the titration curve of the complex implies that the binding of Guo-3′-P to the enzyme stabilizes the molecule of ribonuclease Sa in the His A environment.

The titration curve of His B C(2)-H protons of the free ribonuclease Sa has a very extreme shape. The chemical shifts of the resonance lines of these protons are practically unchanged in the pH range of 4.7—7.5 and it is probable that His B is burried in the molecule and is inaccessible to the titrating agent. We were unable to determine the pK_a of His B C(2)-H protons because of the limited titration range, but we can assume that its value is higher than 8.5. This value is very far from the value for the corresponding parameter of AcHisNHMet. Guo-3′-P does not affect the titration of His B, consequently this nucleotide does not interact with His B and is excluded as a possible component of the active site of ribonuclease Sa.

In an attempt to determine the nature of the interaction of His A with the subsites of Guo-3′-P we investigated the pH dependence of the Guo-3′-P phosphorus chemical shifts in the free nucleotide and in its complex with the enzyme. The titration curves of the ^{31}P atom (i.e. the titration curves of the phosphate group of the nucleotide) are shown in Fig. 6. The formation of the

complex was accompanied by shifts of the resonance lines of ^{31}P and consequently by changes of the pK_a value from 5.94 for the free nucleatide to 7.2 for the complex. These results show that His A of ribonuclease Sa interacts with the phosphate group of Guo-3′-P. This is in agreement with the conclusion derived from our kinetic measurements that in the hydrolysis of Guo-P-Cyd at pH > 7.0 the increase of K_m indicates that the phosphate group of the substrate is bound by a histidine residue of ribonuclease Sa (Both et al. 1982).

Our NMR studies indicate that the active site of ribonuclease differs considerably from active sites of other guanyloribonucleases studied by NMR spectroscopy. While there are many analogous features in the proposed active site models of ribonuclease T_1 (Arata et al. 1979), ribonuclease Pch_1 (Yakovlev et al. 1980) and ribonuclease Pb_1 (Karpeisky et al. 1981) in which 2 histidine residues play a dominant role, the active site of ribonuclease Sa contains only one histidine residue. Further kinetic and NMR experiments are in progress to refine the model of the active site of ribonuclease Sa.

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References

Arata Y., Kimura S., Matsuo H., Narita K. (1976): Proton magnetic resonance studies of ribonuclease T_1 assignment of His-40 peak and analysis of the active site. Biochem. Biophys. Res. Commun. **73,** 133—140

Bezborodova S. I., Bezborodov A. M. (1979): Comparison of exocellular ribonucleases. In: Biosynthesis of Nucleases and Proteases by Microorganisms (Ed. A. A. Imshenetzky), pp. 92—145, Nauka, Moscow (in Russian)

Both V., Witzel H. H., Zelinková E., (1982): Chemical modifications and kinetic study of ribonuclease Sa active site. Gen. Physiol. Biophys. **1**, 261—268

Bradbury J. H., Chapman B. E., Pellegrino F. A. (1973): Hydrogen-deuterium exchange kinetics of the C-2 protons of imidazole and histidine compounds. J. Amer. Chem. Soc. **95,** 6139—6140

Egami F., Takahashi K., Uchida T. (1964): Ribonucleases in Takadiastase: properties, chemical nature and applications. Progr. Nucleic Acid Res. Mol. Biol. **3**, 59—101

Karpeisky M. Ya., Yakovlev G. I., Both V., Ezhov V. A., Prikhodko A. G. (1981): NMR study of the guanylspecific ribonuclease from *Penicillium brevicompactum* fungi. Bioorg. Chim. (Moscow) **7**, 1335—1347 (in Russian)

Roberts G. C. K., Meadows D. H., Jardetzky O. (1969): Nuclear magnetic resonance studies of the structure and binding sites of enzymes. VII. Solvent and temperature effects on the ionisation of histidine residues of ribonuclease. Biochemistry **8**, 2053—2056

Rüterjans H., Pongs O. (1971): On the mechanism of action of ribonuclease T_1. Nuclear magnetic resonance study on the active site. Eur. J. Biochem. **18,** 313—318

Rüterjans H., Maurer R., Fülling R., Both V., (1976): NMR—studies of ribonuclease T_1 and ribonuclease Sa. In: Proceedings of the Second International Symposium on Ribosomes and Ribonucleic Acid Metabolism (Eds. J. Zelinka, J. Balan). pp. 109—118, Veda, Bratislava

Ševčík J.,Gašperík J., Zelinka J. (1982): Amino acid composition, crystallization and preliminary X-ray diffraction studies of guanyloribonuclease from *Streptomyces aureofaciens*. Gen. Physiol. Biophys. **1,** 255—259

Tanokura M., Tasumi M., Miyazawa T. (1976): ^{1}H nuclear magnetic resonance studies at 250 MHz of bovine pancreatic ribonuclease. III. Mutual electrostatic interactions between histidine residues 12 and 119. Biochemistry **14,** 3562—3566

Vaughan J. D., Mughrabi Z., Wu E. C. (1970): The kinetics of deuteration of imidazole. J. Org. Chem. **35,** 1141—1145

Zelinková E., Bačová M., Zelinka J. (1971a): Exocellular ribonuclease from *Streptomyces aureofaciens*. II. Properties and specificity. Biochim. Biophys. Acta **235,** 343—352

Yakovlev G. I., Karpeisky M. Ya., Bezborodova S. I., Beletskaja O. P., Sakharovsky V. G. (1980): Guanyl-specific ribonuclease from the fungus *Penicillium chrysogenum* strain 152 and its complex with guanosine-3'-phosphate studied by nuclear magnetic resonance. Eur. J. Biochem. **109,** 75—85

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