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INTERACTION OF TRANSITION METAL IONS WITH RIBONUCLEASE A

II. THE SELECTIVE EFFECTS OF Mn^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} ON THE HISTIDINE MAGNETIC RESONANCE

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Summary

Zn^{2+}, Cd^{2+} and Hg^{2+} inhibit ribonuclease but Mn^{2+} does not except at very high concentrations. By high resolution NMR one can detect in the pH range 5—8 the C-2 protons of histidines 105, 12, and 119. The inhibiting ions produce large shifts of the resonance of His-12 but not of His-105. On the other hand Mn^{2+} broadens the C-2 proton of His-105 much more than it does those of His-12 and 119. The selective shifts suggest that the mechanism of inhibition is binding at or near the active site of which His-12 and 119 are a part. The selective broadening is a consequence of binding of the Mn^{2+} to a site very far from the active site but closer to His-105.

Introduction

A ribonuclease (EC 3.1.4.22/23) molecule contains 899 protons. Its proton resonance spectrum even at 220 MHz reveals broad bands and only four resolved lines, those of the C-2 protons of the histidines [1]. Even these are seen only at particular pH values. Although proton NMR in general is seen to be a poor tool for the study of ribonuclease it does, in effect, attach three "reporter groups" to the molecule, the protons of histidines 12, 105 and 119. As His-12 and His-119 are part of the active site [2] and His 105 is 20 Å away [3] from the active site (Fig. 1) we obtain simultaneous information about two very different regions of the enzyme. An earlier work from this laboratory [4] studied the broadening of these resonances caused by Cu^{2+} binding. Basically it was found that Cu^{2+} broadened strongly both the C-2 protons of His-105 and His-119 and His-12 about half as much. Thus at least two binding sites for Cu^{2+} had to exist in the protein. X-ray crystallography of RNAase · Cu^{2+} complex

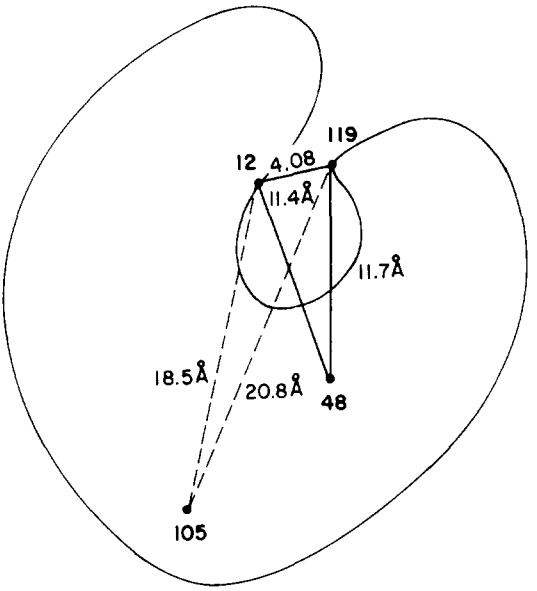

Fig. 1. Side view of the RNAase S' showing the geometry of the four histidine residues. The dotted lines lie behind the plane of His 12, 119 and 48.

[5] shows that Cu^{2+} is bound at a number of places on the surface of the protein including two sites near the above histidines.

In the present work we wished to survey a number of additional ions, some of which are known to inhibit RNAase to see if the proton NMR spectrum could elucidate their inhibition.

Experimental

Materials

Lyophilized and phosphate free RNAase A was obtained from Worthington. This RNAase was dissolved in 99.8% ^2H_2O (Bio-Rad.) overnight to allow the exchangeable N-H protons to become deuterated and then was lyophillized from the ^2H_2O solvent twice. No buffer was used. The weighed amount of RNAase A treated as above was then dissolved in ^2H_2O to give a final concentration of 10^{-2} M. The p^2H of the solution was adjusted using 10 M ^2HCl and NaO^2H solution (from Calbiochem and Stohler Isotope Chemicals).

Anhydrous salts of $MnCl_2$, $CoCl_2$, $NiBr_2$ from K and K Laboratories, $ZnCl_2$, $CdCl_2$ from Fisher and $Hg(NO_3)_2$ from Matheson Coleman and Bell were dried in an evacuated dessicator and ^2H_2O solutions were prepared. Small amounts of rather concentrated salt solutions were added to RNAase A solution to give the desired concentration of the metal ions without altering appreciably the protein concentration and hence its viscosity.

Methods

pH was measured with a Sargent combination microelectrode at room temperature (22—24° C) and with a $KCl/^2H_2O$ solution filled Ag/AgCl refer-

ence electrode. Conversion to p^2H units was made by using the relationship: $p^2H = pH + 0.4$.

NMR spectra were run on a Varian HR 220 MHz spectrometer using 5 mm precision sample tubes (from Wilmad Glass Company). The probe temperature was 18°C determined from the separation of chemical shifts of the CH_3 group and OH proton resonances of pure methanol. The H^2HO residue in the solvent was used as an internal reference. Metal ions at the concentrations used caused no measurable shifts of the H^2HO peaks. Chemical shifts are given both as Hz and ppm downfield from the H^2HO peak (Figs 2—4). The recent spectral assignments of Patel et al. [14] have been used.

p^2H used

A p^2H of about 6.0 was chosen in this study because in this p^2H region, the histidine resonances are maximally separated and the effect of metal ions on them can best be observed. In this range, however, only His-105, 12 and 119 resonances were observed. The absence of the His-48 resonance is due to its extremely broad line width in this p^2H region, thought to be due to a local isomerization of the protein [6,7]. As His-48 is "rather inaccessible" [8], and previous studies with Cu^{2+} showed that this group was not involved in binding, failure to study it in the present work was not considered a major defect. The three resonance peaks of the histidine residues were superimposed on the unexchanged N-H proton resonance region which gave an unevenly shaped broad background. Therefore the measurement of the absolute line width and line shape of the three histidine peaks is very difficult. The baseline chosen for these measurements is not the level of zero absorption, but corresponds to the NH absorption at 810 Hz (3.36 ppm) from the H^2HO peak.

Results

A. Mn^{2+}-RNAase

Measurements were made at p^2H 6.1 with an RNAase A concentration of 0.0123 M. The effects in increasing concentration of Mn^{2+} on the histidine resonances are shown in Fig. 2 which tells us directly what is happening. In going from 2A to 2C, the His-12 and 119 lines are broadened only very slightly but the line width of His-105 has increased from 7 to 24 Hz.

B. Ni^{2+}-RNAase and Co^{2+}-RNAase

The measurement conditions were the same as that in Mn^{2+}-RNAase. From Fig. 2 (A,E,F), the three histidine resonances 105, 12 and 119 showed such a slight broadening at a M^{2+}/RNAase concentration ratio of 1 : 1 that the effect of Co^{2+} and Ni^{2+} on RNAase A can not be studied by proton NMR (or may be negligible in any case).

C. Zn^{2+}-RNAase

A p^2H of 5.9 was used with the RNAase concentration being 0.0128 M. At this p^2H, the HC_4 proton resonance of His-105 was also clearly seen. As shown in Fig. 3, both His-12 and His-119 were shifted downfield while His-105 shifted only slightly upfield. In going from the spectrum in Fig. 3A to 3B, His 105 shifts by +3 Hz, His-119 by 7 Hz and His-12 by —16 Hz. When the Zn^{2+}

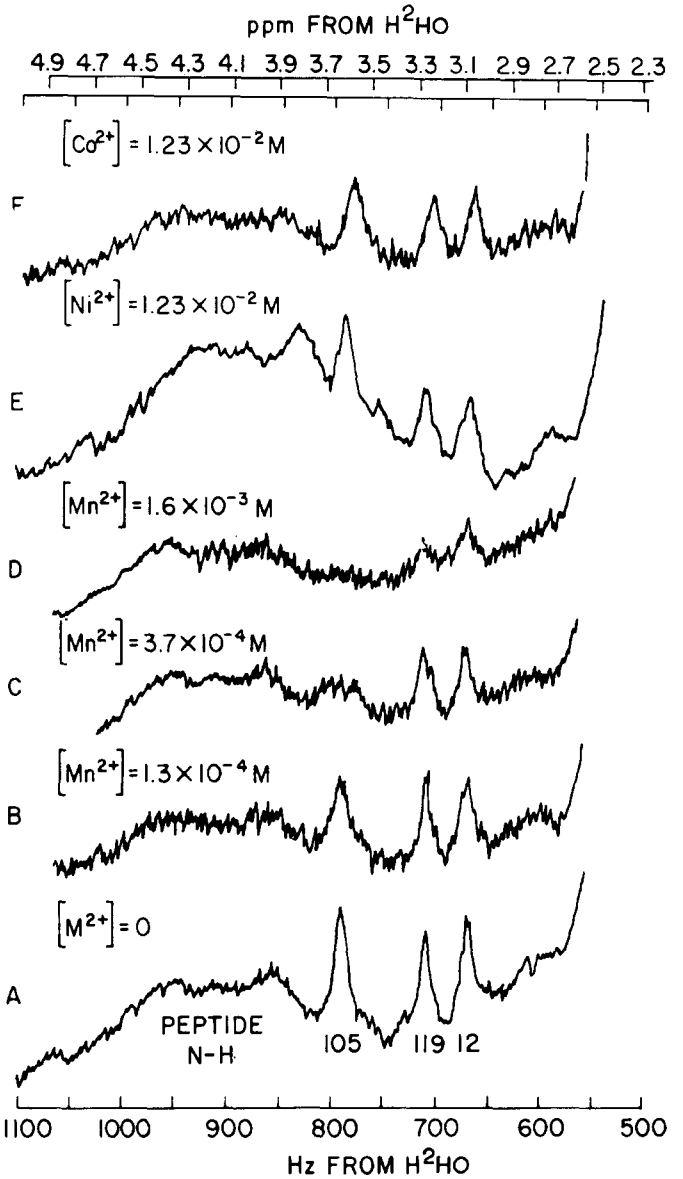

Fig. 2. Effect of Mn^{2+}, Co^{2+}, Ni^{2+} on the histidine resonances of RNAase A at $p^2H = 6.1$.

concentration exceeded $1.3 \cdot 10^{-2}$ M, the His-105 line stayed at the same position and both His-119 and His-12 shifted further downfield. Finally a broad overlapped line is seen.

D. Cd^{2+}-RNAase

The same measurement conditions were used as in the Zn^{2+}-RNAase system. The spectra in Fig. 4 show that Cd^{2+} has an effect on RNAase A similar to the one produced by Zn^{2+} but with the difference that the His-119 line has not been shifted by Cd^{2+}. A comparison of the spectra A, C, D, E shows that as

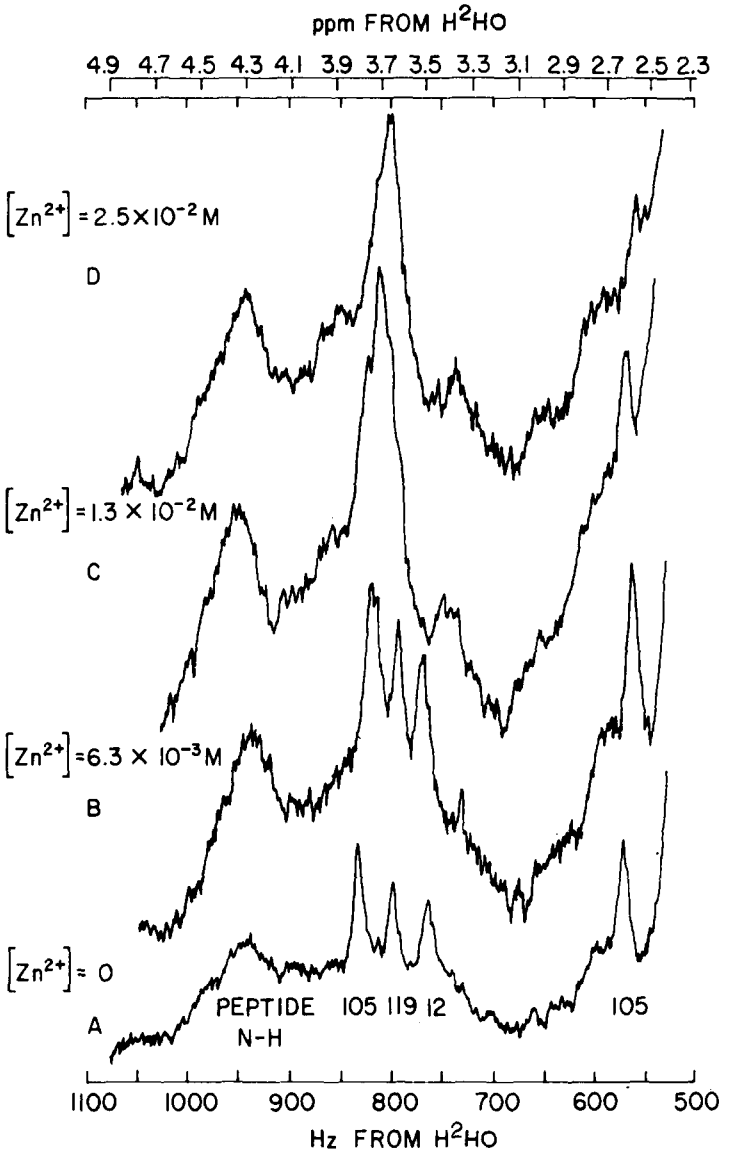

Fig. 3. Effect of Zn^{2+} on the histidine resonances of RNAase A at $p^2H = 5.9$.

Cd^{2+} was added His-12 was shifted downfield from −10 Hz to −32 Hz and His-105 upfield slightly to 5 Hz, while His-119 stayed at the original resonance position. When the concentration of Cd^{2+} exceeded $1.4 \cdot 10^{-2}$ M, His-12 shifted to lower field so much that it coalesced with the His-119 peak.

E. Hg^{2+}-RNAase

Only a concentration of 10^{-5} M Hg^{2+} was used in this experiment. Any attempt to use a higher concentration of this metal ion caused a precipitation of the protein at $p^2H = 5.9$. In Fig. 4, spectra A, B showed that His-105 was

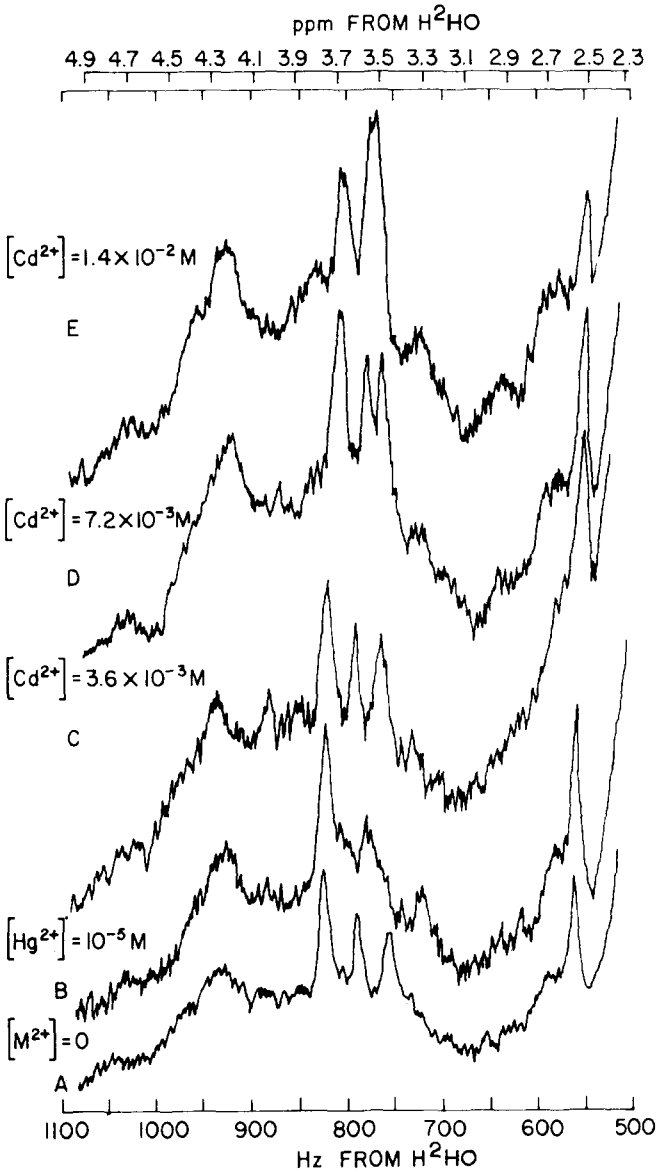

Fig. 4. Effect of Cd^{2+}, Hg^{2+}, on the histidine resonances of RNAase A at $p^2H = 5.9$.

not affected at all while both His-12 and 119 lines were shifted to higher field with the linewidths of both also being broadened. The shift of His-12 is larger than that of His-119.

Discussion

Binding and inhibition

The basic aim of this work has been to find a connection between binding sites and inhibitory properties of the various ions [9—13]. Inhibition depends on conditions such as pH, metal ion concentration and the nature of the substrate to be hydrolyzed. To summarize the literature results, however, both

Cu^{2+} and Zn^{2+} are strong noncompetitive inhibitors of RNAase A irrespective of reaction conditions. Co^{2+}, Ni^{2+}, Ca^{2+} and Hg^{2+} are either weak inhibitors or not inhibitors at all depending upon the reaction conditions. Mn^{2+} is not an inhibitor provided the metal concentration is not greater than the enzyme concentration.

The X-ray crystallographic study of the structure of bovine RNAase S (derived from RNAase A) by Wyckoff et al. has located the position of its four histidine residues whose relative positions are shown in projection in Fig. 1. Wyckoff's statement [8] on the histidines underlies all our results. "His-105 and His-119 are freely accessible, His-12 is partially buried and His-48 is rather inaccessible." From this we expect that there will be basically two binding sites which will involve histidines, the inhibiting site, either 12 or 119 and the noninhibiting site, 105. Binding at either one of the 12, 119 pair which are only 4 Å apart will of course effect the resonance of the other histidines as well.

Figs 2, 3 and 4 together with the numerical data quoted in the Results section allow the following conclusions. When inhibitory ions bind to RNAase without exception, a large effect is produced on the HC_2 resonance of either the 12 or the 119 histidine or both. When the non-inhibitory ion Mn^{2+} binds to RNAase there is a much larger effect on the His-105 resonance than on the active site histidines showing that its binding site is much nearer the 105 site than the active site.

In conclusion we have found four kinds of behavior of ions with the two ^1H NMR observable sites of RNAase A. A strong inhibitor like Cu^{2+} broadens strongly the resonances at both sites. A strong inhibitor like Zn^{2+} shifts strongly the resonances at the active site but not at the 105 site. Very weak inhibitors like Ni^{2+} and Co^{2+} cause no effect at the 105 site and only a weak effect at the active site. Finally Mn^{2+} which binds but does not inhibit the enzyme except at high concentrations has its strongest binding site relatively far from the active site. Inhibition is accompanied by large changes at the active site presumably caused by binding there. Binding to the protein, on the other hand, does not produce inhibition if, as with Mn^{2+}, the binding site is far from the active site.

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