ASSIGNMENT OF THE C-2 HISTIDINE PROTON MAGNETIC RESONANCES OF RIBONUCLEASE-A

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Received May 19,1972;revised September 20,1972

SUMMARY

Deuteration of ribonuclease-A (RNase-A) at pH 8.5 and 37° for 5 days produces differential exchange of the four C-2 histidine proton resonances numbered 1 (downfield), 2, 3, 4 (upfield). Resonances 1 and 2 are almost completely removed whereas resonances 3 and 4 retain 35% and 26% of their original areas respectively. The partially deuterated RNase-A is separated into the S-peptide (residues 1-20) which lacks a C-2 proton resonance and the S-protein (residues 21-124) which shows two resonances. This result gives unequivocal confirmation of the assignment of Meadows et al. (1), viz. that resonances 1,2,3,4 correspond to histidines 105, 12, 119 and 48 respectively.

The histidine residues in RNase-A occur at positions 12, 119 (active site histidines), 48 and 105 in the sequence and give rise to four C-2 proton resonances (1-6), one from each C-2 proton in the molecule. The assignment of the four proton resonances, number 1 (downfield), 2, 3, 4 (upfield) (1,3), was made in three steps. (a) The proton resonances numbered 1 and 4 are not affected by addition of inhibitors and hence correspond to histidine residues 105 and 48. The assignment of resonance 4 to His 48 and resonance 1 to His 105 is firmly based on the correlation between the magnetically perturbed nature of resonance 4 (1,4-7) and the buried histidine, His 48 (8,9) and between the unperturbed nature of resonance 1 with a surface histidine, His 105 (8,9). (b) The resonance 2 and 3 are assigned to His 12 and His 119 respectively in ribonuclease-S (RNase-S), by comparison of the spectra and

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titration curves of a normal sample of RNase-S and another in which the C-2 proton of His 12 is replaced by deuterium (1). (c) The extrapolation from the unequivocal assignment of RNase-S to RNase-A involves the assumption that the titration curves of His 12 and His 119 are both moved to lower pH values by about 0.5 units (1), rather than the alternative that one changes very much and the other hardly at all (6). Because of the obvious importance for mechanistic studies (9,10) of a correct assignment, we have checked and confirmed the original assignment (1) by direct deuterium exchange of RNase-A.

EXPERIMENTAL

RNase-A (Worthington Biochemicals, lyophilised and phosphate free) was used to prepare RNase-S and the S-peptide and S-protein by a published procedure (11). The H-D exchange of RNase-A was carried out at 37^{o} in an unbuffered 1% solution of enzyme in D_2O at a pH meter reading of 8.5, with regular adjustment of the pH (all pH readings are recorded as such). At appropriate times samples were removed, the solvent lyophilised off and a 10% solution of the enzyme prepared at the appropriate pH for study by proton magnetic resonance (PMR) spectroscopy. The spectra were obtained at 100 MHz using a JEOL MH-100 PMR spectrometer on line to a PDP8/S computer which was used for spectrum accumulation (normally about 100-300 scans were averaged). The C-2 histidine resonances of RNase-A and derivatives were observed in D_2O solutions at a pH of 5.7. Under these conditions the upfield resonance (number 4) due to His 48 is not visible (4-7), but the other three resonances are well separated. The His 48 resonance was observed by reducing the pH to 4 and running a second spectrum. The number of protons contributing to each C-2 histidine resonance was obtained by comparing the weight of the tracing of the peak with the weight of the large aromatic resonance which contained a known number of protons.

RESULTS AND DISCUSSION

The H-D exchange of the C-2 histidine protons of RNase-A is shown

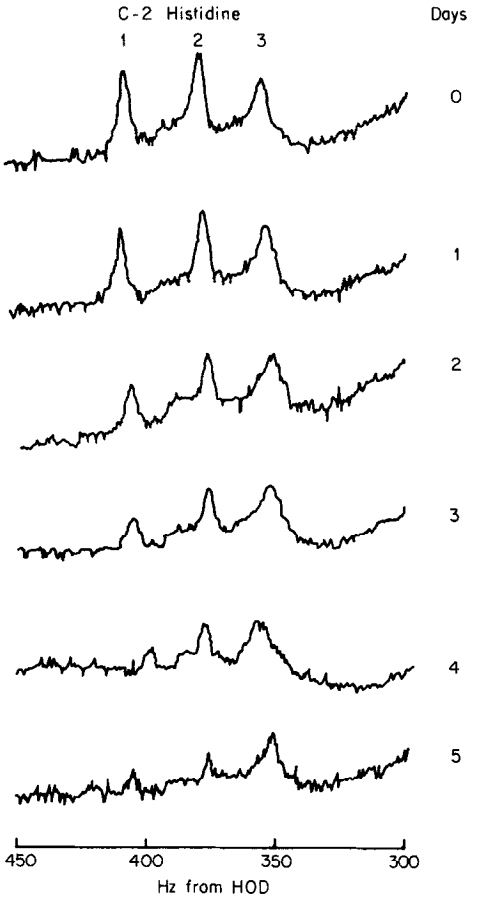

Figure 1. Exchange of RNase-A with D_2O at 37° and pH 8.5 over 0-5 days as shown by the P.M.R. spectra of C-2 His protons 1, 2, 3, which were measured at pH 5.6 in D_2O. Resonance 4 is not observable at this pH.

in Figure 1, and one example of the observation of resonance 4, after 4 days of exchange, is shown in Figure 2. The rate of exchange of the different C-2 protons with deuterium is clearly different. The first order rate constants for the process are readily calculated from the straight line graphs in Figure 3 to be 0.48, 0.48, 0.19, 0.25 $days^{-1}$ for resonances 1,2,3 and 4 respectively. These rate constants can be rationalised on the basis of exchange studies made on model compounds (12). After 5 days deuteration, the area of peaks 1, 2, 3 and 4 were found to correspond to 0.07, 0.08, 0.35 and 0.26 protons respectively.

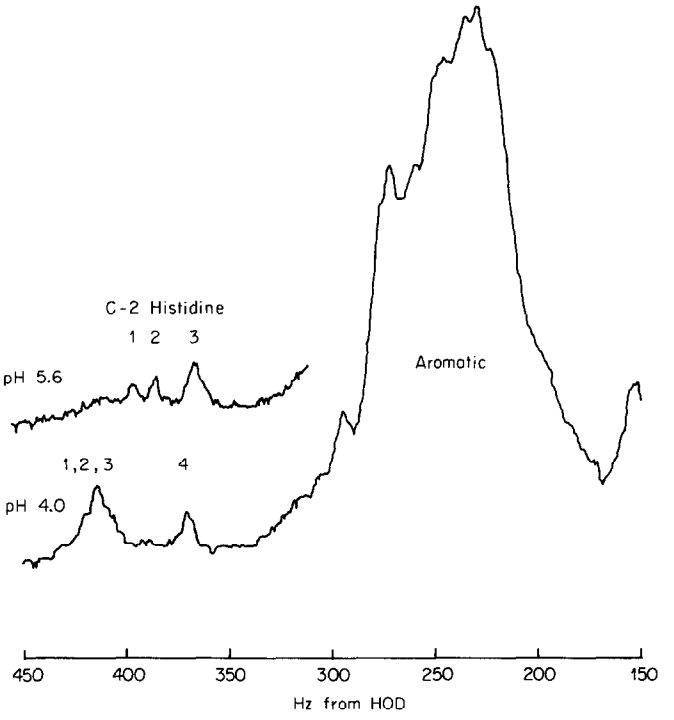

Figure 2. PMR spectra of RNase-A after 4 days exchange in D_2O at 37° and pH 8.5. The spectra obtained at pH 4 contains resonance 4 (His 48) which is absent in the spectra at pH 5.6.

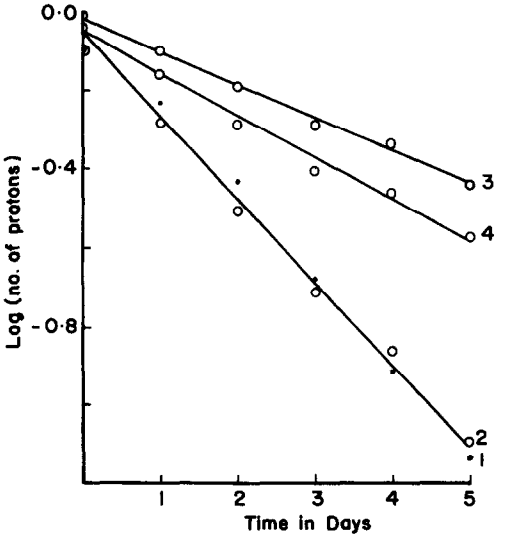

Figure 3. Graph of log (number of protons in C-2 resonance) vs time of exchange in D_2O at 37° and pH 8.5, for the C-2 resonances 1, 2, 3, 4.

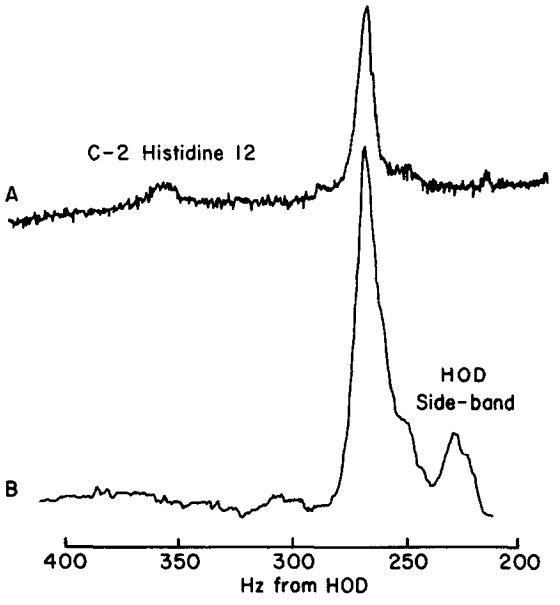

Figure 4. PMR spectra of S-peptide prepared from RNase-A (ref. (1)) (A) and from RNase-A after deuteration for 5 days (B). S-peptide spectra determined on 2% solution in 0.1 M acetate buffer in D_2O at pH 7 and 10°.

This sample and another one of native RNase-A were each separated into their S-peptide (residues 1-20) and S-protein (residues 21-124) fractions. at 5° to prevent back exchange. As shown in Figure 4 the S-peptide fragment from the deuterated RNase-A gives no evidence of a C-2 histidine resonance as compared with the spectrum of the C-2 peptide from native RNase-A (1). Thus His 12 corresponds to either resonance 1 or 2, each of which have very low residual areas. However resonance 1 is known from the work of Meadows et al. (1) to correspond to His 105. Also the active site histidines must correspond to resonances 2 and 3 (1,3) hence resonance 2 is assigned to His 12.

The spectrum of the S-protein of RNase-A shown in Figure 5a shows three C-2 histidine resonances (labelled a, b and c) with a total area of 3.2 protons compared with the large aromatic resonance. The deuterated S-protein sample gives only 2 resonances b and c of areas 0.35 and 0.40 protons respectively (Figure 5b). It is clear that resonance "a" which disappears

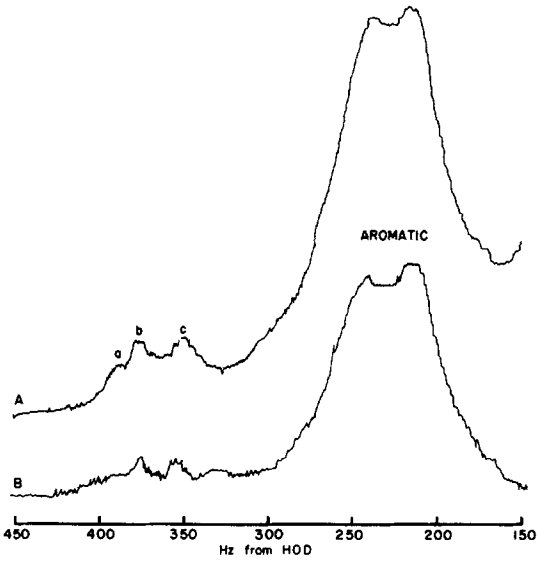

Figure 5. PMR spectra of S-protein prepared from native RNase-A (A) and RNase-A deuterated for 5 days (B). S-protein spectra determined in 10% solutions in 0.1 M acetate buffer at pH 5.9.

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on deuteration corresponds to His 105, resonance b corresponds very nicely on an area basis (0.35 protons in both RNase-A and the S-protein) with His 119 and resonance c. (His 48) has a predicted area of 0.26 protons but gives an observed value of 0.40 protons (possibly due to excessive noise in the peak in Figure 5B).

The spectra of the S-protein therefore confirm the conclusion obtained from those of the S-peptide, viz. that resonances 2 and 3 correspond with His 12 and 119 respectively, and show that there is no possible alternative explanation. We therefore conclude that the original assignment of Markley et al. (1) is correct and that the C-2 resonances 1 (downfield, 2, 3, 4 (upfield) of RNase-A correspond to histidines 105, 12, 119 and 48 respectively.

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