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A ^{31}P MAS NMR study of cytidine 2′-phosphate bound to ribonuclease A in the crystalline state

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^{31}P CP/MAS spectra have been obtained from 2′-CMP bound to ribonuclease A in the crystalline state. The chemical shift value is closely similar to that found in solution NMR studies under similar conditions, and corresponds to that of the dianionic state of the free compound. It is suggested that the NMR approach may be of general applicability for the comparison of the binding properties of small molecules to proteins in crystals and solution.

RNase A; ^{31}P- NMR; Cytidine 2′-phosphate; Solid-state NMR

1. INTRODUCTION

NMR spectroscopy has been used for many years to investigate the structure, dynamics and function of biological molecules in solution [1,2]. Recently it has become possible using the techniques of magic angle spinning and high power decoupling to obtain high-resolution NMR spectra of samples in the solid state [3]. This has enabled extension of NMR studies to biological systems such as membranes and viruses that cannot satisfactorily be studied in solution [4–9]. In addition, the possibility of obtaining high-resolution spectra at low temperatures [10] where solution spectra of even relatively small molecules are usually broad offers opportunities to investigate species trapped on reaction pathways.

A somewhat different aspect of the solid-state NMR techniques is that they can enable, in conjunction with solution NMR, a direct comparison of the behaviour of molecules in the solid and solution states [11–13]. This is of major interest in studies of proteins where structural data are obtained in the crystalline state and need to be related to functional properties in solution. Of central importance in such cases are studies of the binding of small molecules, such as substrates or inhibitors, which provide a structural basis for mechanistic descriptions [11]. Here, we describe the results of an investigation by cross-polarization magic angle spinning (CP/MAS) ^{31}P NMR of the binding of cytidine 2′-phosphate (2′-CMP) to RNase A which provides an example of the potential of solid-state NMR studies of this type.

RNase is an enzyme which has been studied extensively by X-ray diffraction, and a number of complexes of RNase with nucleotides have been characterized [14–16]. Recent studies of a series of these have been used to provide a detailed description of the structural changes in both enzyme and substrate that occur during catalysis [16]. The enzyme has also been studied extensively in solution by NMR and of particular interest here are ^{31}P NMR results which relate to the ionization states of bound inhibitors [17,18]. In the case of 2′-CMP the chemical shift of the bound phosphate group

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was observed to be very close to that of the dianionic state of 2′-CMP in free solution, a result rationalized on the basis of interactions with Lys 41 and His 119 in the active site [17]. Below pH 7, therefore, a substantial shift in the ^{31}P resonance takes place on binding as under these conditions free 2′-CMP will be present in the monoanionic form. The CP/MAS NMR experiments described here have been designed to investigate whether a similar shift is observed on binding 2′-CMP to crystalline RNase under conditions similar to those where diffraction studies have been carried out, hence permitting direct comparison between binding in the solution and crystalline states.

2. MATERIALS AND METHODS

RNase A was obtained from Sigma and crystals were prepared as described by Gorinsky [19]. Ethanol was added slowly whilst gently stirring to a 37% (w/w) solution of RNase in water at 5°C, pH 5.5, until the mixture was slightly cloudy. The ethanol-water protein mixture was allowed to stand at room temperature and seeded after 24 h. Crystals for NMR spectroscopy were gathered after 3–4 weeks.

Co-crystallisation of RNase with the inhibitors was carried out using the above method except that the protein was dissolved in a 5-fold excess of the inhibitor solution at pH 5.5. An alternative method used to obtain 2′-CMP bound to RNase in crystals was to soak the protein crystals for 24 h in an ethanol-water solution containing a 5-fold excess of 2′-CMP at the desired pH value. This procedure was monitored by X-ray crystallography and confirmed that the inhibitors were bound to the protein in the manner described in [15].

The crystals of the protein-inhibitor complex were washed with an ethanol-water solution at the same pH value at which the crystals were grown (or soaked). The fresh crystals were dried in air prior to acquisition of NMR spectra. Typically, between 25 and 50 mg of each sample was used in each solid-state NMR experiment. Due to the small quantities involved, each sample was packed between 2 layers of an inert material. KBr was used here as it was also employed in adjusting the magic angle [20]; to prevent contamination of the sample by KBr the layers were kept separate using filter paper. Spectra were recorded at room temperature on a Bruker CXP 200 spectrometer operating at 80.96 MHz for ^{31}P and 200.13 MHz for ^{1}H. Magic angle spinning was carried out using either Delrin Andrew-type rotors or ceramic rotors using a double-bearing probe. Typical spinning speeds ranged from 2.2 to 3.2 kHz and all spectra were accumulated using high-power proton decoupling. For the cross-polarization experiments the Hartmann-Hahn condition was met using a B_1 field of 10 G for ^{1}H and 24.7 G for ^{31}P. The optimum contact time for cross-polarization was found to range from 1.0 to 2.5 ms, depending on the sample. A reasonable S/N ratio was obtained after about 10000 scans. Solution-state NMR spectra were recorded on the Bruker AM 500 spectrometer of the Leicester Biological NMR Centre. Samples were prepared by dissolving appropriate amounts of RNase and 2′-CMP in water, adding ethanol to produce a 30% solution, and adjustment of the pH to the desired value. A higher proportion of ethanol resulted in precipitation or crystallization. Both the solution and solid-state spectra were referenced externally to 80% orthophosphoric acid at 0 ppm.

3. RESULTS AND DISCUSSION

As the RNase crystals used here were grown from ethanol-water solutions, an initial study was made of the binding of 2′-CMP to RNase in solution under conditions similar to those used for the crystallisation experiments. Fig.1 shows ^{31}P NMR spectra of 2′-CMP in the presence of 0.5 mole equivalents of RNase. Two distinct resonances are observed at pH values between 4 and 7.5, and a concentration dependence allowed the low-field resonance to be attributed to 2′-CMP bound to the active site of the protein. Previous studies of 2′-CMP binding to RNase in aqueous solution have observed only an averaged signal because of rapid exchange between the free and bound nucleotide [17,18]. The slower exchange conditions observed here were found to be partly a consequence of the high magnetic field used in the present experiments but primarily from slower kinetics in the ethanol-water mixture.

The pH dependence of the spectra (figs 1,2) shows that the resonance of unbound 2′-CMP titrates with a pK value of 6.3 ± 0.2, close to the value of 5.95 found for 2′-CMP in aqueous solu-

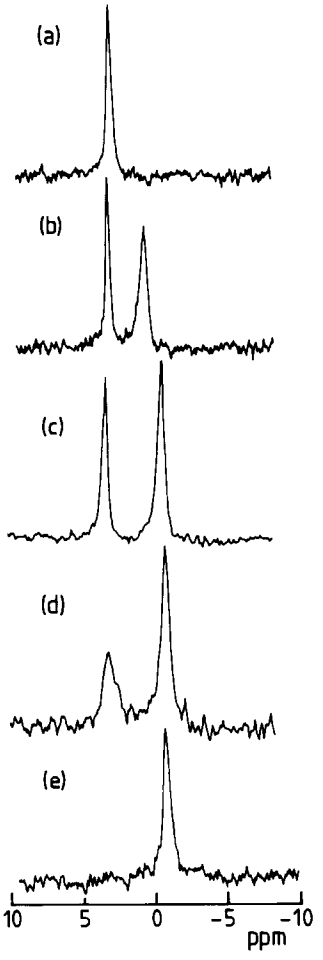

Fig.1. 202.4 MHz ^{31}P NMR spectra at 25°C of 30% ethanol/70% water solutions containing 6 mM 2′-CMP and 3 mM RNase. The pH values were (a) 8.13, (b) 6.40, (c) 5.41, (d) 4.18 and (e) 3.15.

tion [17]. The bound resonance, however, shows no change in chemical shift between pH 4.0 and 7.5, a result consistent with the previous studies in aqueous solutions. Below pH 4, however, the resonance of the bound 2′-CMP shifts markedly. In aqueous solution a similar shift in the averaged resonance of the free and bound nucleotide was attributed primarily to the dissociation of the bound molecule at low pH values [17]. The slow exchange conditions prevailing in the ethanol-water mixtures, however, show that the resonance of the bound nucleotide shifts to a value corresponding to the monoanionic state observed for 2′-CMP in

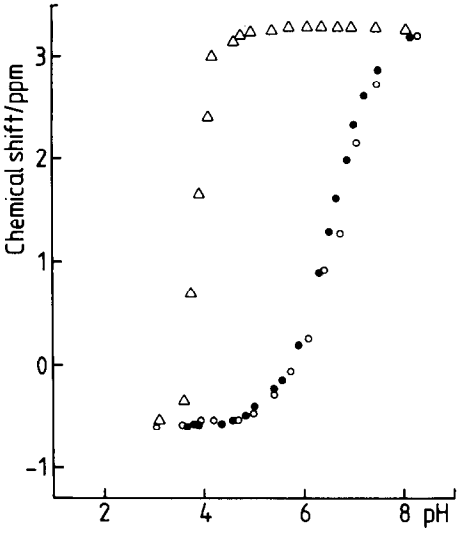

Fig.2. pH dependence of the chemical shifts of 2′-CMP in 30% ethanol/70% water solution. (△,○) Points from an experiment such as that shown in fig.1, corresponding to the resonances of the bound and unbound states, respectively. (●) Points of 2′-CMP under similar conditions but in the absence of RNase.

solution, the midpoint for the titration occurring at pH 4.0 ± 0.2. The change in chemical shift with pH is, however, much steeper than for a simple ionization process [17]. One possible explanation for this is that ionization of the cytidine base, which in solution has a pK value close to 4.0, alters the binding of 2′-CMP and hence the protonation state of the bound phosphate group. The major conclusion, however, is that as in aqueous solution, bound 2′-CMP in ethanol-water solutions above pH 4.0 has a chemical shift corresponding to the dianionic state of the phosphate group.

^{31}P CP/MAS NMR spectra of RNase crystals soaked in 2′-CMP under different conditions are shown in fig.3. For the crystals soaked at pH 7.1, a single line is observed; the linewidth of ~1 ppm is close to that which we have obtained for the pure crystalline nucleotides themselves. The chemical shift is very close to the value found for bound 2′-CMP in solution. At pH 5.5, however, the peak was reproducibly much broader, and markedly asymmetric with a shoulder to high field. Comparison with the solution spectra of fig.1 shows that although the observed shift is still close to that characteristic of the dianionic form of the

phosphate group, the possibility of a significant contribution to the signal from 2′-CMP with a shift closer to that of the monoanionic state cannot be eliminated.

There is little doubt that the only species observed in the spectra are those bound to the protein; no cross-polarization is anticipated for species such as 2′-CMP dissolved in solvent present within the crystals. Further, the concentration of any such species appears to be very small as no signal could be detected from the crystals in experiments carried out without cross-polarization. At least two possibilities must, however, be considered. One of these is that, unlike the solution case, 2′-CMP bound in the active site in the crystals is present in more than one environmental or ionization state at pH 5.5. Another is that there is occupancy of one or more secondary binding sites in the crystals which bind 2′-CMP as the monoanion at pH values where this is the dominant species in solution. Evidence that the latter is the probable explanation comes from experiments involving crystals prepared in the presence of pyrophosphate.

^{31}P CP/MAS NMR spectra of RNase crystals prepared at pH 5.5 from solutions containing pyrophosphate revealed a broad resonance at 8.1 ppm, close to the chemical shift of pyrophosphate bound to RNase in solution [17]. When these crystals were soaked in 2′-CMP under conditions otherwise identical to those used to prepare the samples described above, two narrow resonances were observed, readily assigned to 2′-CMP and pyrophosphate (see fig.3c). This shows that both compounds are bound in the RNase crystals. Given that the binding constant for 2′-CMP in the active site is 10^4 greater than for pyrophosphate in solution under these conditions [21], 2′-CMP is likely to displace pyrophosphate from this site after soaking the crystals for 24 h in excess 2′-CMP. In accord with this, the resonance of 2′-CMP in fig.3c is narrow and lies at 3.8 ppm, very close to the chemical shift found for the phosphate group bound in the active site in solution. The shoulder to high field is absent, showing no evidence for the binding of 2′-CMP in a different state in the active site or indeed elsewhere. The differences between the spectra at pH 5.5 in the presence and absence of pyrophosphate could arise if the bound pyrophosphate, clearly detected

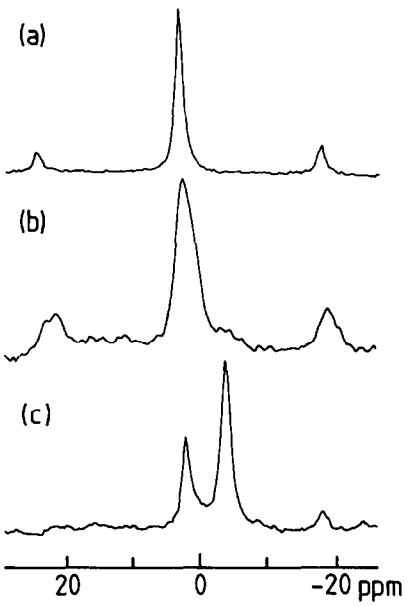

Fig.3. 80.96 MHz CP/MAS ^{31}P NMR spectra of 2′-CMP in RNase crystals obtained by soaking at (a) pH 7.1, (b) pH 5.5 and (c) pH 5.5 using crystals prepared in the presence of pyrophosphate.

in the NMR spectra, represents binding to sites other than the active site from which it is not displaced by 2′-CMP. Although the intensities of resonances in CP spectra must be interpreted with caution, the 2:1 ratio between those of the pyrophosphate and 2′-CMP suggest that approx. 1 mol phosphate is bound per mol bound 2′-CMP, which would be consistent with a single secondary binding site occupied by pyrophosphate under these conditions. Regardless of whether or not this is the correct interpretation of this result the experiment indicates strongly that 2′-CMP is bound under these conditions in a manner identical to that observed in solution and with a chemical shift corresponding to that of the dianionic state of unbound 2′-CMP.

This study has demonstrated one approach to using CP/MAS NMR to investigate an enzyme-small molecule complex in a crystal, and to compare properties of the solution and crystalline states. ^{31}P is a particularly favourable nucleus for such a study because it has high sensitivity and natural abundance, yet problems with resolution and assignment are avoided. Further, the atoms present are frequently in chemical groups which

are ionizable and involved in metal ion binding, and which have functional roles. We have applied this approach to other proteins in both crystalline and freeze-dried states, and obtained good spectra of even large proteins such as phosphorylase *b* [22]. Extension of these studies to low temperatures should allow the investigation of enzyme-substrate complexes, and the study of the anisotropy of chemical shifts should be of value in understanding dynamic aspects of such complexes.

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REFERENCES

[1] Jardetzky, O. and Roberts, G.C.K. (1981) NMR in Molecular Biology, Academic Press, New York.
[2] Wüthrich, K. (1986) NMR of Proteins and Nucleic Acids, Wiley, New York.
[3] Fyfe, C.A. (1983) Solid State NMR for Chemists, CFC Press, Guelph.
[4] Opella, S.J. (1986) Methods Enzymol. 131, 327–361.
[5] Diverdi, J.A. and Opella, S.J. (1982) J. Am. Chem. Soc. 104, 1761–1762.
[6] Munowitz, M., Dobson, C.M., Griffin, R.G. and Harrison, S.C. (1980) J. Mol. Biol. 141, 327–333.
[7] Harbison, G.S., Smith, S.O., Pardoen, J.A., Mulder, P.P.J., Lugtenberg, J., Herzfeld, J., Mathies, R. and Griffin, R.G. (1984) Biochemistry 23, 2662–2667.
[8] Roufosse, A.H., Aue, W.P., Roberts, J.E., Glimcher, M.J. and Griffin, R.G. (1984) Biochemistry 23, 6115–6120.
[9] Colnago, L.A., Valentine, K.G. and Opella, S.J. (1987) Biochemistry 26, 847–854.
[10] Smith, S.O., Palings, I., Copie, V., Raleigh, D.P., Courtin, J., Pardoen, J.A., Lugtenburg, J., Mathies, R.A. and Griffin, R.G. (1987) Biochemistry 26, 1606–1611.
[11] Mackenzie, N.E., Fagerness, P.E. and Scott, A.I. (1985) J. Chem. Soc. Chem. Commun., 635–637.
[12] Huang, T.-H., Bachovchin, W.W., Griffin, R.G. and Dobson, C.M. (1984) Biochemistry 23, 5933–5937.
[13] Clayden, N.J., Dobson, C.M., Lian, L.-Y. and Twyman, M. (1986) J. Chem. Soc. Perkin II, 1933–1940.
[14] Richards, F.M., Wyckoff, H.W., Carlson, W.D., Allewell, N.M., Lee, B. and Mitsui, Y. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 35–43.
[15] Borkakoti, N. (1983) Eur. J. Biochem. 1329, 89–94.
[16] Fink, A.L., Gilbert, W.A. and Petsko, G.A., to be published.
[17] Haar, N., Thompson, J.C., Mauver, W. and Ruterjans, H. (1973) Eur. J. Biochem. 40, 259–266.
[18] Gorenstein, D.G., Wyrwicz, A.M. and Bode, J. (1976) J. Am. Chem. Soc. 98, 2308–2314.
[19] Gorinsky, B.A. (1971) PhD Thesis, Birkbeck College, University of London.
[20] Frye, J.S. and Maciel, G.E. (1982) J. Magn. Reson. 48, 125–131.
[21] Richards, F.M. and Wykoff, H.W. (1971) in: The Enzymes (Boyer, P.D. ed.) 3rd edn, vol.4, pp.647–806, Academic Press, New York.