Straightforward detection of the secondary ionisation of the phosphate group and p*K* determinations by high-resolution solid-state ^{31}P NMR†

Carole Gardiennet,[a] Bernard Henry,[a] Paul Kuad,[b] Bernard Spiess[b] and Piotr Tekely*[a]

Received (in Cambridge, UK) 2nd September 2004, Accepted 23rd September 2004
First published as an Advance Article on the web 25th October 2004
DOI: 10.1039/b413415j

The suitability of high-resolution solid-state ^{31}P NMR for a straightforward determination of the protonation state of phosphate groups as well as of their pK_2 values extracted from solid state mono : dianionic ratios has been demonstrated.

There is still a good deal of effort directed towards the chemistry involving phosphate groups which are known to play a crucial role in life sustaining processes.[1] The phosphate groups display two main p*K* values. In the primary ionisation state, one proton is removed, resulting in a single negative charge per phosphate group. This state can prevail up to a pH of around 7 where the remaining proton is removed in the secondary ionisation step. One of the most important issues when analysing kinases and phosphatases at the molecular level or the activation of biomolecules is whether a phosphate group is mono- or dianionic. The ionisation state of phosphate groups may also depend, or be related to, conformational changes of phosphorylated proteins.[2]

In this communication, we show for the first time that solid-state ^{31}P NMR spectroscopy yields an unambiguous visualisation of the presence and the nature of single and/or double ionised phosphate groups in lyophilizates prepared from parent solutions at different pH values. For this, we have chosen three phosphorylated model compounds, each of them being of prime importance in the chemistry of enzymatic and cellular regulation.[1] Moreover, some of us have shown very recently that solid-state, natural abundance ^{13}C NMR determinations of the acid–base ratios of lyophilised compounds permit the measurement of the p*K* values of the successive deprotonations, without recourse to full titration curves.[3] Herein, we will show that the calculated p*K* values for a secondary phosphate ionisation, available from acid–base ratios in solid-state ^{31}P NMR spectra, are also found to be remarkably similar to those classically measured in the liquid state.

Fig. 1 shows the high-resolution cross-polarization/magic-angle spinning (CP/MAS) ^{31}P NMR spectrum of D-*myo*-inositol 2-monophosphate [Ins(2)P_1] lyophilisate prepared from solution at pH = 6.13. Although the easiest way to record the quantitative ^{31}P NMR spectrum is *via* direct excitation by a single pulse, this cannot always be applied due to excessively long ^{31}P longitudinal relaxation times. As shown in Fig. 1, and contrary to the liquid state ^{31}P NMR spectra, two isotropic chemical shifts are present simultaneously at 1.96 and 5.13 ppm and are assigned respectively to single and double deprotonated phosphate groups according to the observed shifts of the resonance signal in solution at different pH values.[4] The simultaneous presence of well-separated, isotropic peaks for mono- and dianionic species in the lyophilizate shows a dramatic slowing down of inter- and intramolecular proton exchanges on the NMR timescale.[3] From the ratio of the integrated intensity of the resonance signals, including the corresponding spinning sidebands (not shown in Fig. 1), and taking into account somewhat different $T_{1\rho}$ (^{1}H) relaxation decays of single and double deprotonated species on the kHz frequency scale, the calculated solid state[3] pK_2 values for samples prepared at a slightly different pH values, change between 5.58 and 5.72. The same range of values has been obtained directly from the single pulse excitation spectra. This is indeed very close to the solution pK_2 value of 5.83 (Et_4NCLO_4, 0.1 M, 25 °C).[4]

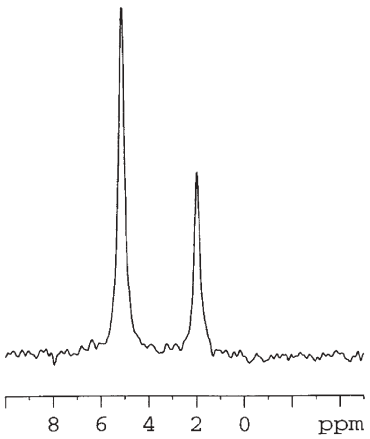

Fig. 1 ^{31}P isotropic chemical shifts in the solid-state CP/MAS NMR spectrum of D-*myo*-inositol 2-monophosphate lyophilisate prepared from solution at pH = 6.13 and recorded at 121.46 MHz, CP contact time of 1 ms and a spinning frequency of 5 kHz.

A clear spectral separation of the ^{31}P isotropic chemical shifts, as observed in Fig. 1, is not however systematic, and in the case of smaller chemical shift differences and/or larger resonance line-widths of differently ionized species, only a single, featureless resonance line can be observed at different pH values. In spite of these complicating factors, one can obtain the desired information by taking advantage of another benefit of solid-state measurements resulting from easy access to the principal values of chemical shift anisotropy (CSA) tensors. The chemical shielding interaction is indeed of prime interest for chemists involved in the study of molecular structure or dynamics and is appropriately described by a tensor whose nature depends upon the local electronic environment of the nucleus and its local site symmetry. In

† Electronic supplementary information (ESI) available: ^{31}P NMR values of the CSA tensor elements and corresponding parameters for L-*o*-phosphoserine and AMP-5′ as a function of pH. See http://www.rsc.org/suppdata/cc/b4/b413415j/

*Piotr.Tekely@rmn.uhp-nancy.fr

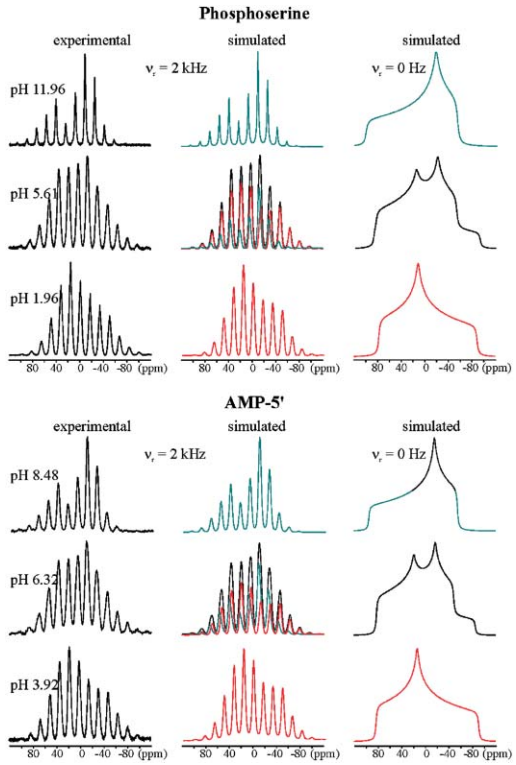

Fig. 2 Experimental (left) and simulated (middle) low spinning speed (ν_r = 2.0 kHz) CSA sideband manifolds of phosphate groups in L-*o*-phosphoserine and AMP-5′ lyophilized from solutions at different pH values. For better visualisation of the changes in the CSA tensor after secondary ionisation, the corresponding simulated static powder spectra are also included (right).

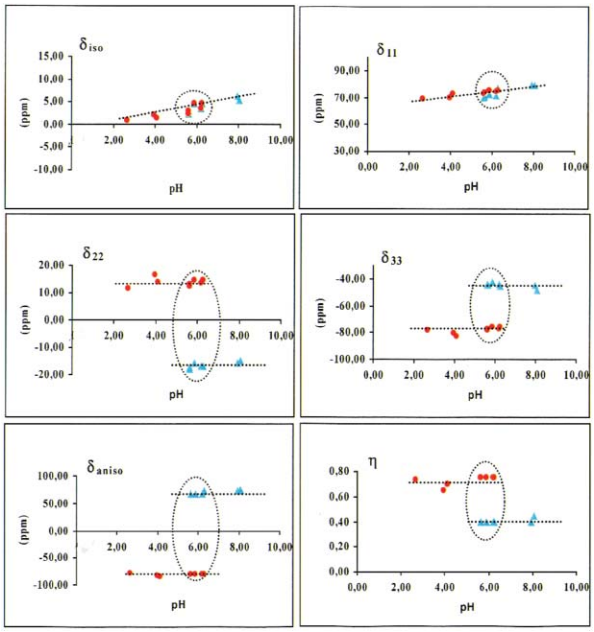

Fig. 3 Changes of the isotropic chemical shift δ_{iso}, the principal values δ_{ii} as well as the anisotropy (δ_{aniso}) and asymmetry (η) parameters of the ^{31}P CSA tensor of the phosphate group in L-*o*-phosphoserine as a function of the pH. The dotted circles and ellipses cover the pH range of the secondary ionisation transition. The anisotropy and asymmetry parameters have their usual meaning (see Electronic Supplementary Information†).

solution, the molecular tumbling averages out the spectral features due to the anisotropic part of the chemical shielding tensor, leaving its isotropic part $\delta_{iso} = (\delta_{11} + \delta_{22} + \delta_{33})/3$, and where δ_{ii} are three principal elements of the chemical shift tensor. As shown in Fig. 1, a rapid magic-angle spinning of powder solids also leads to an averaged isotropic value for a given resonance. However, by virtue of their nature, the principal components are more sensitive to the changes in the ionisation state[5] and provide much more detailed information in relation to zwitterionic structure and intermolecular contacts than the isotropic part.[6]

Fig. 2 shows ^{31}P CP/MAS (CT = 1 ms) spectra of L-*o*-phosphoserine and of adenosine 5′-monophosphate (AMP-5′) lyophilizates prepared at different pH values (adjusted with NaOH at 25 °C). In both cases, remarkably similar envelopes of spinning sidebands have been recorded when going from the monoanionic, low pH, phosphate groups to the dianionic phosphates at a higher pH. This must be due to the characteristic effect of the ionisation state of the phosphate group on the form of the ^{31}P CSA tensor. The principal elements δ_{ii}, anisotropy (δ_{aniso}) and asymmetry (η) of the ^{31}P CSA tensors in mono- and dianionic phosphate groups were derived from the simulations of corresponding spinning sideband manifolds. The changes of these spectroscopic features as a function of the pH are reported in Fig. 3 for L-*o*-phosphoserine (for exact values see Electronic Supplementary Information†).

Fig. 3 shows that when going to higher pH values, in contrast to a continuous increase of δ_{iso} and δ_{11}, the second ionisation is accompanied by a dramatic jump in opposite directions of both δ_{22} and δ_{33} values leading themselves to the jumps of anisotropy and asymmetry values. This is directly related to the individual sensitivity of each principal element resulting from the particular orientation of the ^{31}P chemical shielding tensor with respect to the molecular frame of the phosphate group.[6]

From the simulations of spinning sideband manifolds in the range of secondary ionisation where both mono- and dianionic species are present simultaneously, we have determined the ratios of the amounts of both forms, which as demonstrated earlier,[3] allow the calculation of the p*K* values. For *o*-phosphoserine and AMP-5′, respectively, the average pK_2 values of 5.9 and 6.43 have been obtained; these are only slightly higher than the corresponding average values of 5.72 and 6.18 measured in solution.

As in the ^{13}C NMR determinations, we can assign such a difference to temperature effects,[3] since the freezing out of aqueous solutions begins at temperatures close to 0 °C, whereas the liquid state titrations were conducted at 25 °C. However, further factors like the small changes in the initial concentrations as well as the nature of the counterions must be considered and are currently under thorough investigation.

Summing up, we have shown that solid-state ^{31}P NMR allows a direct visualisation of the characteristic spectroscopic features related to single and double ionized phosphate groups in lyophilizates prepared from parent solutions at different pH values. Calculated pK_2 values from solid-state mono : dianion ratios were found to be very close to those classically measured in solution. We anticipate numerous applications of such a straightforward approach answering the question of whether a phosphate group is mono- or dianionic.

Carole Gardiennet,[a] Bernard Henry,[a] Paul Kuad,[b] Bernard Spiess[b] and Piotr Tekely*[a]
[a]*UMR CNRS 7565, Université Henri Poincaré, Nancy 1, 54506 Vandoeuvre-lès-Nancy, France.*
E-mail: Piotr.Tekely@rmn.uhp-nancy.fr; Fax: +33 383684347; Tel: +33 383684352
[b]*UMR CNRS 7081, Faculté de Pharmacie, Université Louis Pasteur, 67401 Illkirch, France*

Notes and references

1 F. H. Westheimer, *Science*, 1987, **235**, 1173; L. N. Johnson and R. J. Lewis, *Chem. Rev.*, 2001, **101**, 2209; S. M. Jones and A. Kazlauskaz, *Chem. Rev.*, 2001, **101**, 2413; A. J. Bridges, *Chem. Rev.*, 2001, **101**, 2541; R. Majeti and A. Weiss, *Chem. Rev.*, 2001, **101**, 2441.
2 J. M. Antosiewicz, J. A. McCammon and M. K. Gilson, *J. Mol. Biol.*, 1994, **238**, 415; E. Blachut-Okrasinska, B. Lesyng, J. M. Briggs, J. A. McCammon and J. M. Antosiewicz, *Eur. Biophys. J.*, 1999, **28**, 457.
3 B. Henry, P. Tekely and J. J. Delpuech, *J. Am. Chem. Soc.*, 2002, **124**, 2025; R. Colsenet, C. Gardiennet, B. Henry and P. Tekely, *Angew. Chem., Int. Ed.*, 2002, **41**, 4743.
4 K. Mernissi-Arifi, L. Schmitt, G. Schlewer and B. Spiess, *Anal. Chem.*, 1995, **67**, 2567.
5 Y. Wei, A. C. de Dios and A. J. McDermott, *J. Am. Chem. Soc.*, 1999, **121**, 10389.
6 M. P. Potrzebowski, X. Assfeld, K. Ganicz, S. Olejniczak, A. Cartier, C. Gardiennet and P. Tekely, *J. Am. Chem. Soc.*, 2003, **125**, 4223.

