Investigation of the inframolecular acid–base properties of d-*myo*-inositol 1,3,4,5-tetrakisphosphate and dl-*myo*-inositol 1,2,4,5-tetrakisphosphate

Philippe Guédat,[a] Gilbert Schlewer,[a] Elisabeth Krempp,[a] Andrew M. Riley,[b] Barry V. L. Potter[b] and Bernard Spiess[a,b]

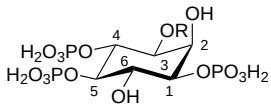
[a] *Laboratoire de Pharmacochimie Moléculaire, UPR 421 du CNRS, Faculté de Pharmacie, 74, route du Rhin, B.P. 24, 67401 Illkirch Cedex, France*

[b] *Department of Medicinal Chemistry, School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, UK BA2 7AY*

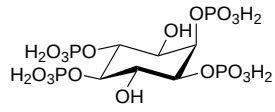
The protonation processes of the individual phosphate groups in two regioisomeric inositol polyphosphates, D-*myo*-inositol 1,3,4,5-tetrakisphosphate and DL-*myo*-inositol 1,2,4,5-tetrakisphosphate, have been investigated by ^{31}P NMR titration experiments; the results are compared to those from D-*myo*-inositol 1,4,5-trisphosphate.

d-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] **1** (Fig. 1) produced in response to stimulation of a wide variety of cellular membrane receptors, exhibits well-known second messenger properties.[1,2] Besides Ins(1,4,5)P_3 a host of inositol polyphosphates, among them d-*myo*-inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] **2**, have also been characterised in the cytoplasm of many cell types. While the role of Ins(1,4,5)P_3 and its calcium mobilising ability is well-defined in many tissues, the physiological role of Ins(1,3,4,5)P_4 remains a matter of much debate and controversy. In some cases Ins(1,3,4,5)P_4 potentiates the Ins(1,4,5)P_3-induced response,[3,4] while in others it inhibits calcium signalling.[5] A main route to resolving the precise function of Ins(1,3,4,5)P_4 is to identify the molecular nature of its receptor[6,7] and recently a specific Ins(1,3,4,5)P_4 binding protein has been characterised and denoted as GAP1^{IP4BP}.[8] A complementary method is to focus on the ligand itself and to compare its physico-chemical properties with those of Ins(1,4,5)P_3. In this respect, Ins(1,2,4,5)P_4 **3**, a regioisomer of Ins(1,3,4,5)P_4 and a potent analogue of Ins(1,4,5)P_3,[9,10] is also worthy of consideration. A fundamental difference in the binding of both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 to their specific or putative receptors respectively is the effect of pH. The affinity of the former for its receptor is increased by raising the pH,[11] whereas the inverse occurs for the latter.[6] A consideration of the acid–base properties of these ligands is of prime importance in gaining reliable knowledge of the binding mechanisms and we have therefore chosen for investigation two regioisomeric inositol tetrakisphosphates, differing only in the substitution position of a single phosphate group, but with strikingly different biological properties.

Herein we present the protonation constants as well as a picture of the protonation process of the individual phosphate groups for Ins(1,3,4,5)P_4 and Ins(1,2,4,5)P_4 (IP4s). The results will further be compared to the acid–base behaviour of Ins(1,4,5)P_3.[12,13] The study was performed in 0.2 mol dm^{-3} KCl at 37 °C, a medium that mimics the ionic strength and temperature conditions encountered in the cell.



R = H, Ins(1,4,5)P_3 **1**
R = PO_3H_2, Ins(1,3,4,5)P_4 **2**



Ins(1,2,4,5)P_4 **3**

Fig. 1 Structure of inositol polyphosphates (d-isomers are shown)

The logarithms of the stepwise protonation constants for the studied ligands (L^{n-}), K_y, referring to the equilibria $H_{y-1}L^{(n-y+1)-} + H^+ \Leftrightarrow H_yL^{(n-y)-}$ are listed in Table 1. In comparison with the constants determined for Ins(1,4,5)P_3,[13] an additional phosphate group raises the first protonation constant by two orders of magnitude. This is clearly the result of the increase in negative charge density on the *myo*-inositol ring phosphates that electrostatically stabilises the binding of the first proton. However, by considering the values in Table 1, it can be observed that both IP4s display nearly the same overall basicities, Ins(1,3,4,5)P_4 showing only a slightly more pronounced basic character over Ins(1,2,4,5)P_4. While a simple examination of these macroscopic protonation constants seems to indicate a similar acid–base behaviour it is therefore necessary to investigate the protonation process of the two IP4s at an 'inframolecular' level, *i.e.* at each individual phosphate group.

In a previous work[14] we showed that ^{31}P NMR spectroscopy gives easy access to the protonated fraction $f_{i,p}$ of a phosphate group in position *i* on the inositol ring if the observed chemical shift for the resonance δ_i^{obs} depends on the electronic effects accompanying the deprotonation of this group. The value of $f_{i,p}$ can then be calculated from eqn. (1),

$$f_{i,p} = \frac{\delta_i^{obs} - \delta_{i,d}}{\delta_{i,p} - \delta_{i,d}} \quad (1)$$

where $\delta_{i,p}$ and $\delta_{i,d}$ correspond to the chemical shifts of the protonated and deprotonated fractions of the phosphates in position *i*, respectively. The previous condition can be checked by the superimposition of the $\bar{p} = f(pH)$ curves calculated from both the potentiometric and NMR data according to eqns. (2) and (3), respectively.

$$\bar{p} = \frac{C_H - [H^+] + [OH^-]}{C_L} \quad (2)$$

Table 1 Logarithms of the stepwise protonation constants for d-Ins(1,3,4,5)P_4 and dl-Ins(1,2,4,5)P_4 in KCl 0.2 mol dm^{-3} (H_2O) at 37 °C[a]

log K_y	Ins(1,4,5)P_3	Ins(1,3,4,5)P_4	Ins(1,2,4,5)P_4
y = 1	7.85	9.92 ± 0.02	9.39 ± 0.02
y = 2	6.40	7.66 ± 0.02	7.65 ± 0.02
y = 3	5.31	6.36 ± 0.02	6.06 ± 0.02
y = 4	–	5.23 ± 0.02	5.23 ± 0.02
$N(n)$	–	4(221)	2(198)

[a] These constants were determined by potentiometry (see ref. 12) where the pH measurements are processed by the program SUPERQUAD (ref. 16). The constants are the result of N experiments including a total number of n observations. The standard deviations are given by SUPERQUAD. The values for Ins(1,4,5)P_3 were taken from ref. 13.

$$\bar{p} = \sum_{i=1}^{i=N} f_{i,\mathrm{p}} \qquad (3)$$

In these eqns., $\bar{p}$ is the mean number of protons bound per mole of IP4 and C_H and C_L correspond to the analytical concentrations of the acid and IP4, respectively.

For Ins(1,3,4,5)P_4 and Ins(1,2,4,5)P_4 both potentiometric and NMR $\bar{p} = f(\mathrm{pH})$ curves superimpose well from pH 2 to 8.5 (curves not shown). Above this pH a slight difference is noted indicating that δ_i^{obs} comprises, in addition to the protonation effect, another effect presumably due to conformational changes. Consequently, the $f_{i,\mathrm{p}} = f(\mathrm{pH})$ curves strictly describe $f_{i,\mathrm{p}}$ below pH 8.5 and give a good approximation of it above this pH.

The $f_{i,\mathrm{p}} = f(\mathrm{pH})$ curves for the studied IP4s are shown in Fig. 2. It can be seen that, even though the overall basicities of the IP4s are similar, the protonation fraction curves differ markedly indicating distinct protonation processes. Ins(1,2,4,5)P_4 exhibits almost monophasic (P1 and P2) and biphasic (P4 and P5) curves which resemble those of the corresponding bisphosphates. The general pattern of these curves attests to the independence of the two bisphosphate moieties. However, compared to Ins(1,4,5)P_3, the basicities of P4 and P5 appear inverted. The $f_{i,\mathrm{p}} = f(\mathrm{pH})$ curves for Ins(1,3,4,5)P_4 are much more complicated due to the strong interaction between phosphates P3, P4 and P5. As expected, P3 and P5 behave similarly, whereas P4, which experiences the highest density of negative charge, shows a very peculiar protonation process already observed for P5 in Ins(4,5,6)P_3.[15] The monophasic appearance retained by the P1 curve attests to the independence of this phosphate group. Consideration of the protonation fraction curves allows a detailed investigation of the protonation state of each individual phosphate group at any pH. Thus, at a near physiological pH (7.50), for Ins(1,3,4,5)P_4 and Ins(1,2,4,5)P_4 the percentage of protonated phosphates are P1 (5%), P3 (50%), P4 (60%), P5 (60%) and P1 (60%), P2 (15%), P4 (70%), P5 (35%), respectively. Large differences can be observed, especially in the basicity of P1 and P5 which are common to both IP4s. The observation that P5 is much more basic for Ins(1,3,4,5)P_4 than Ins(1,2,4,5)P_4 may be of biological significance. Indeed, it has been suggested that the increase in affinity of Ins(1,4,5)P_3 for its receptor when the pH rises may be caused by the ionization of the P5 phosphate group.[12] Therefore, binding of Ins(1,2,4,5)P_4 to the Ins(1,4,5)P_3 receptor could also be favoured by a low protonation state of the P5 phosphate at physiological pH. In constrast to Ins(1,4,5)P_3, for Ins(1,3,4,5)P_4, specific binding decreases steadily from pH 5.5 to 9.0. Moreover, these specific binding data *vs*. pH could be roughly superimposed on the P3 and P5 NMR titration curves (*e.g*. at pH 7.5, $f_{5,\mathrm{p}}$ *ca*. 60% and specific binding *ca*. 65%).[6] It is therefore likely that, if the protonation state of Ins(1,3,4,5)P_4 is involved in its binding to a specific protein, the observed high basicity of P5 will undoubtedly advantage such a binding. The results described herein which demonstrate the acid–base requirements needed for the observation of either an Ins(1,2,4,5)P_4 type or an Ins(1,3,4,5)P_4 type of biological activity may be of great help to chemists who aim at the synthesis of potent analogues. Further studies on the influence of the ionic environment of the IP4s on their acid–base and conformational properties are in progress and will be reported in due course.

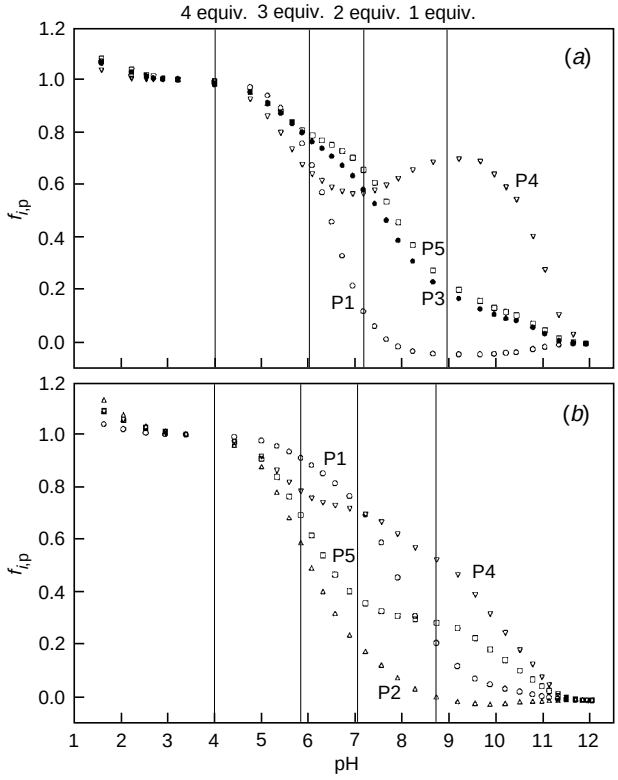


Fig. 2 Protonation fraction curves $f_{i,\mathrm{p}}$ as a function of pH in KCl 0.2 mol dm^{-3} (99.9% D_2O) at 37 °C for d-Ins(1,3,4,5)P_4 (*a*) and d1-Ins(1,2,4,5)P_4 (*b*). The vertical lines correspond to the theoretical addition of 1–4 equiv. of protons. These curves were calculated according to eqn. (1) from ^{31}P NMR titrations carried out at 121.497 MHz on a Bruker 300 DPX FT spectrometer. Chemical shifts were measured relative to an external 85% orthophosphoric reference. Resonance peaks of d-Ins(1,3,4,5)P_4 and d1-Ins(1,2,4,5)P_4 were assigned by performing proton phosphorus 2D correlation experiments at pH 1.62, 2.25, 7.92, 12.00 and 1.65, 5.93, 7.90, 11.65, respectively. The assignment of the proton resonances was done on the basis of the chemical shifts and the coupling patterns confirmed by ^1H–^1H COSY experiments. The titrations were performed as previously indicated[14] but on a volume of 0.45 ml instead of 2 ml. Ins(1,2,4,5)P_4 was prepared as described in ref. 9 and the synthesis of Ins(1,3,4,5)P_4 will be published elsewhere.

References

1 M. J. Berridge, *Nature*, 1993, **361**, 315.
2 M. J. Berridge, *Ann. N. Y. Acad. Sci*., 1996, 31.
3 L. B. Zhang, M. E. Bradley and I. L. O. Buxton, *Int. J. Biochem. Cell Biol*., 1995, **27**, 1231.
4 J. W. Loomishusselbee, P. J. Cullen, U. E. Dreikhausen, R. F. Irvine and A. P. Dawson, *Biochem. J*., 1996, **314**, 811.
5 G. S. J. Bird and J. W. Putney, *J. Biol. Chem*., 1996, **271**, 6766.
6 P. J. Cullen, S. K. Chung, Y. T. Chang, A. P. Dawson and R. F. Irvine, *FEBS Lett*., 1995, **358**, 240.
7 B. Huisamen and A. Lochner, *Mol. Cell. Biochem*., 1996, **157**, 229.
8 P. J. Cullen, J. J. Hsuan, O. Truong, A. J. Letcher, T. R. Jackson, A. P. Dawson and R. F. Irvine, *Nature*, 1995, **376**, 527.
9 M. Hirata, N. Narumoto, Y. Watanabe, T. Kanematsu, T. Koga and S. Ozaki, *Mol. Pharmacol*., 1994, **45**, 271.
10 R. A. Wilcox, S. T. Safrany, D. Lampe, S. J. Mills, S. R. Nahorski and B. V. L. Potter, *Eur. J. Biochem*., 1994, **223**, 115.
11 P. F. Worley, J. M. Baraban, S. Supattapone, V. S. Wilson and S. H. Snyder, *J. Biol. Chem*., 1987, **262**, 12 132.
12 L. Schmitt, P. Bortmann, G. Schlewer and B. Spiess, *J. Chem. Soc., Perkin Trans. 2*, 1993, 2257.
13 L. Schmitt, G. Schlewer and B. Spiess, *Biochim. Biophys. Acta*, 1991, **1075**, 139.
14 K. Mernissi Arifi, L. Schmitt, G. Schlewer and B. Spiess, *Anal. Chem*., 1995, **67**, 2567.
15 L. Schmitt, B. Spiess and G. Schlewer, *Bioorg. Med. Chem. Lett*., 1995, **5**, 1225.
16 P. Gans, A. Sabatini and A. Vacca, *J. Chem. Soc., Dalton Trans*., 1985, 1195.

Received, 12th December 1996; Com. 6/08347A