myo-Inositol 1,4,5,6-Tetrakisphosphate and *myo*-Inositol 3,4,5,6-Tetrakisphosphate, Two Second Messengers that May Act as pH-Dependent Molecular Switches

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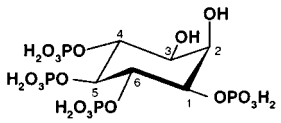
Since *myo*-inositol 3,4,5,6-tetrakisphosphate (Ins(3,4,5,6)P_4) was designated as an "orphan" messenger[1] in 1996, many studies have explored its metabolism and physiological functions in the intestinal epithelial cell line T_{84}.[2,3] In these cells and in others, Ins(3,4,5,6)P_4 is responsible for the inhibition of calcium-mediated chloride secretion[1,4−6] which may have therapeutic implications for disease states of cystic fibrosis and secretory diarrhea.[6]

In contrast, *myo*-inositol 1,4,5,6-tetrakisphosphate (Ins(1,4,5,6)-P_4) shows distinct biological properties. For instance, Ins(1,4,5,6)-P_4 at micromolar concentrations inhibits IGF-1-induced [^{3}H]-thymidine incorporation in human breast cancer cells and thus prevents the ability of these cells to grow.[7] It is noteworthy that while Ins(1,4,5,6)P_4 is able to almost completely inhibit the response induced by IGF-1, Ins(3,4,5,6)P_4 only shows 25% inhibition.[7] Recent studies have revealed that Ins(1,4,5,6)P_4 is also involved in nuclear processes since it is required for gene regulation and may control gene expression.[8] Thus, a new area of design of anticancer drugs can be envisaged for the future.[9]



Ins(1,4,5,6)P_4 and Ins(3,4,5,6)P_4 are enantiomers[10] that play distinct but critical roles in cellular signaling. Their mechanisms of action are far from being elucidated but appear to be closely related to the number and position of the phosphate groups on the inositol ring. In addition, the complex electrolyte movements occurring in the intracellular medium and in proximity to the cell membrane are very likely to be involved in the biological mechanism of such highly charged polyanionic ligands. As for other previously investigated inositol tetrakisphosphates of biological interest,[11,12] the acid−base, complexation, and conformational properties of these ligands may be of prime importance in understanding the binding mechanisms and regulation processes.

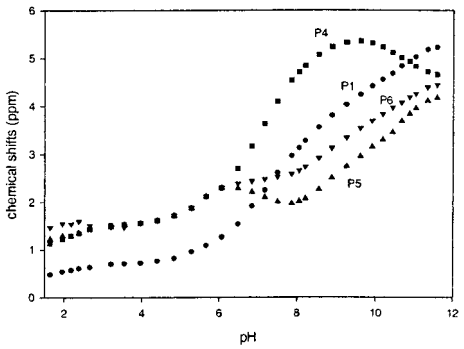


Figure 1. Chemical shifts δ from ^{31}P NMR titrations at 37 °C.

Using ^{1}H and ^{31}P NMR titration experiments we present herein the inositol ring conformational dependence on the ionization state of the phosphate groups for an inositol tetrakisphosphate carrying four vicinal phosphates in the same configuration (Ins(1,4,5,6)P_4 or Ins(3,4,5,6)P_4).

Figure 1 shows ^{31}P NMR titration curves obtained in 0.2 M KCl at 37 °C. As expected, upon deprotonation of the phosphate groups, the resonances of the phosphorus nuclei generally move downfield, with the exception of P4 and P5 which, in two distinct pH areas, undergo a shift in the opposite direction, referred to as the "wrongway" shift.[13] Such unusual shifts have been attributed either to transfer of protons from the lateral phosphates to the central phosphate, reflecting a protonation process even though this protonation occurs while the pH increases,[14] or to the establishment of a C−H···O hydrogen bond between a phosphate oxygen constrained to closely approach a neighboring equatorial hydrogen atom.[13] The upfield shift observed for P5 in the 6−8 pH range results from the former effect, whereas the final wrongway shift for P4 cannot be attributed to either of these effects. In particular, this 0.7 ppm shift in the unexpected direction cannot be the consequence of an interaction with the axial H3 hydrogen atom. To shed light on this new kind of wrongway shift, the titration experiments were repeated at −15 °C at the same ionic strength in a 70/30 D_2O/CD_3OD medium. From the ^{31}P NMR curves displayed in Figure 2, it is apparent that the final P4 upfield shift arises from a *myo*-inositol ring flip from a conformation that contains four phosphates in an equatorial position (4eq) to a conformation where these phosphates are inverted (4ax). Such a conformational switching occurs at both temperatures around pH 9−10, but cannot be discerned at 37 °C due to a high rate of interconversion of the two conformers. However, by comparing the chemical shifts of both curves, it appears that even for the highest temperature, 4ax is the predominant conformer at the end of the titration.

P. P. N. Murthy et al.[15] thoroughly investigated the conformational preferences of inositol mono to hexakisphosphates by ^{1}H NMR. They observed pH-dependent ring conformational changes for the inositol pentakis and hexakisphosphates, but not for the inositol tetrakisphosphates that they studied, that is, Ins-(1,2,3,4)P_4 and Ins(1,2,5,6)P_4 which do not display four equatorial

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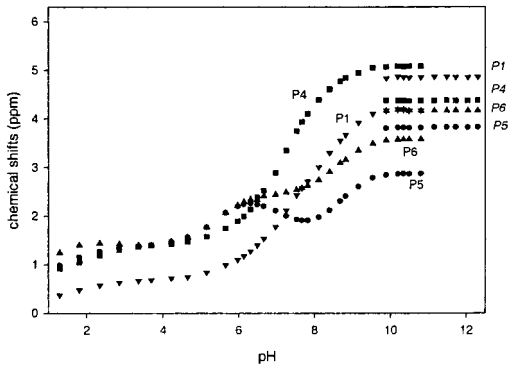
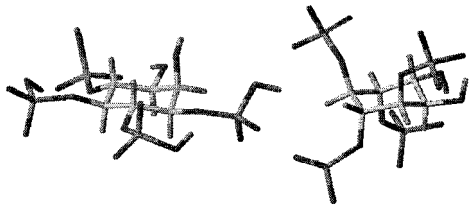


Figure 2. Chemical shifts δ from ^{31}P NMR titration at −15 °C in a 70/30 D_2O/CD_3OD medium.

vicinal phosphates. The latter condition seems critical to the flipping of the inositol ring.[15] The curves of Figure 2 clearly show that the 4eq to 4ax conformational change is triggered by complete deprotonation of all phosphate groups. Although presumably sterically hindered, the 4ax conformer minimizes electrostatic repulsion between the vicinal dianionic phosphates. Hydration and binding of metallic cations by the phosphates may additionally influence ring flipping.

To gain insight into the energetics of the ring-flipping process, molecular modeling studies were performed using the same algorithms and molecular parameters as previously used for Ins(1,4,5)P_3.[13] Upon removing four protons from optimized 4eq tetrahydrogeno Ins(1,4,5,6)P_4 conformer, thus giving the fully deprotonated Ins(1,4,5,6)P_4, the energy of the ring-constrained species was found to be 77 kcal·mol^{-1}. When the constraint is removed, mutual repulsions of the phosphate groups lead to a twisted boat inositol ring with 64 kcal·mol^{-1} of energy, conformation that is not in accord with the experimental observations. However, by starting from the 4ax conformer, the energy decreases to 54 kcal·mol^{-1}, confirming the tendency of the four contiguous dianionic phosphates to adopt an axial configuration. Both minimized tetrahydrogeno (4eq) and deprotonated (4ax) forms of Ins(1,4,5,6)P_4 are shown below.



4eq **4ax**

To confirm the conclusions drawn from the ^{31}P NMR titration curves, the corresponding ^{1}H NMR titration was performed (Figure 3). On going from the lowest to the highest pHs, all of the resonances, except that of H2, undergo a sudden downfield shift around pH 10, indicating conversion of axial protons to equatorial ones.

As expected, all of the proton resonances moved upfield to varying extents upon deprotonation. Interestingly, H2 in the 4eq conformer is downfield-shifted by 0.18 ppm from pH 8 to 10

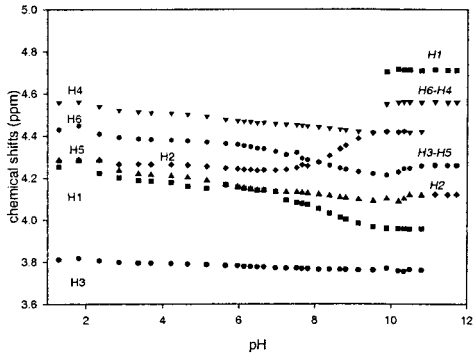


Figure 3. Chemical shifts δ from ^{1}H NMR titration at −15 °C.

due to the formation of a C−H···O hydrogen bond between H2 and P1 as already observed for Ins(1,4,5)P_3[13] and related compounds.[16] This is undoubtedly the sign of strong electrostatic repulsion in the nearly deprotonated 4eq form, repulsion which is suddenly canceled when switching into the 4ax form.

The biological relevance of this conformational change deserves further analysis. Only one conformer is expected to be present at the binding site of the receptor. However, if the free form of this conformer is in equilibrium with its inverted form, every factor that affects this equilibrium will also modulate the binding to the receptor and therefore the resulting physiological response. From Figure 1 it can be seen that the deshielding of P4 starts at pH 9, suggesting that the 4ax conformer becomes predominant above this pH, but also that it still may be significantly present from pH 8 onward. In addition, it must be stressed that the protonation constants of inositol polyphosphates containing several vicinal phosphate groups vary by several orders of magnitude according to the concentration of alkali or alkali-earth cations present in the surrounding medium.[17] Since the 4eq ⇌ 4ax equilibrium of Ins(1,4,5,6)P_4 is governed by the ionization state of the phosphate groups which itself strongly depends on the ionic environment, it is very likely that the major changes in concentration of cations that occur in proximity to the receptor will modulate the equilibrium between both conformers.

The participation of conformational changes in Ins(1,4,5,6)P_4 or Ins(3,4,5,6)P_4 in their complex mechanism of action has so far not been proven. Since substitution of these compounds on position 2 is tolerated and preserves their biological activity,[18] the synthesis of fluorescent probes with distinct spectroscopic characteristics according to the conformation of the inositol ring can be envisaged. Such molecules would be important sensors able to detect conformational flipping modulated both by the ionization state of their phosphate groups and their ionic environment.

Supporting Information Available: ^{31}P and ^{1}H NMR spectra referring to Figures 1−3 (PDF). This material is available free of charge via the Internet at http:/pubs.acs.org.

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