Journal of Molecular Structure 986 (2011) 75–85

Contents lists available at ScienceDirect

Journal of Molecular Structure

journal homepage: www.elsevier.com/locate/molstruc

Insight into the protonation and K(I)-interaction of the inositol 1,2,3-trisphosphate as provided by ^{31}P NMR and theoretical calculations

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ARTICLE INFO

Article history:
Received 27 September 2010
Received in revised form 19 November 2010
Accepted 19 November 2010
Available online 28 November 2010

Keywords:
Inositol polyphosphates
Iron
Chemical speciation
Potassium
Theoretical calculations

ABSTRACT

Animal cells contain a pool of inositol phosphates whose biological function is still under current investigation. Ins(1,2,3)P_3 is probably an important safe chelator of iron cations not strongly bound to proteins. In order to clarify its biological functions, Ins(1,2,3)P_3 chemistry under physiological conditions must be completely elucidated. The protonation and complexation behaviour of Ins(1,2,3)P_3 has been recently studied under these conditions by potentiometry. Under simulated physiological conditions it forms the protonated species H_2L^{4-} and H_3L^{3-}. The presence of high concentrations of potassium in intracellular compartments causes the formation of two predominant Ins(1,2,3)P_3 complexes: $[K(HL)]^{4-}$ and $[K(H_2L)]^{3-}$, in the absence of iron. In this work we expand part of this macroscopic knowledge to the inframolecular level, by ^{31}P NMR measurements and focusing on the protonation and complexation of this biologically relevant molecule to potassium. We complete this study with theoretical calculations which lead us to predict the geometries of every form of the ligand and their relative stabilities. The influence of the ring conformation in protonated and complexed forms is also discussed.

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1. Introduction

myo-inositol phosphates (Ins*P*s) are a wide range of biomolecules derived from the phosphorylation of *myo*-inositol. This group contains more than 25 metabolically related species, differing only in the number and position of the phosphate groups attached to the inositol ring. Even though they are ubiquitous and abundant in eukaryotic cells, their functions are still scarcely understood [1]. Only one of them, *myo*-inositol 1,4,5 trisphosphate, has reached the textbooks with a specific biological function related to intracellular calcium mobilization [2].

Up to now, clarification of the biological functions of the Ins*P*s has been hindered by their intricate structural and metabolic interrelated characteristics. Over the past years, we have carried out a systematic study of the unusual and often non-intuitive chemical behaviour displayed by these highly charged compounds, in particular in the presence of multivalent cations [3–8]. These reports have shed light over the acid–base and complexation equilibria established by InsP_6, Ins(1,3,4,5,6)P_5 and Ins(1,2,3)P_3 in various biological systems. These data have provided relevant information about the overall charge of the species under simulated biological compartmental conditions, setting up the basis for explaining some physicochemical, structural, and biological properties of these molecules.

The inframolecular acid–base properties together with the microscopic interaction between each inositol phosphate and the biologically relevant metal ions are expected to be involved in the biological activity of these compounds. In fact, it is important to investigate the structural requirements of the interaction among the biologically relevant Ins*P* species, the metal ions and the proteins they regulate. This will help not only to the understanding of the main biological processes at the molecular level but also to the rational design of agonists and antagonists with therapeutic value. Thus, a thorough investigation of the microprotonation and metal coordination processes and its structural and conformational consequences may be one of the key issues in gaining reliable knowledge of the biological roles of Ins*P*s.

The overwhelming majority of the equilibrium constants of Ins*P*s protonation and interaction with metal ions reported so far correspond to macroscopic protonation constants and only characterize the molecule as a whole. There are few studies focusing on the protonation state at an inframolecular level and/or the intrinsic

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0022-2860/$ - see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.molstruc.2010.11.050

metal complexation properties of *myo*-inositol phosphates [9–12]. In particular for Ins(1,2,3)P_3, a molecule whose biological function is still controversial, some information regarding the intrinsic acid–base properties has been reported [12], although the data were acquired in the presence of large amounts of K(I), which substantially interacts with this ligand in weakly acid to basic media [4]. This is expected to alter the ^{31}P NMR signals and, consequently, the macro and micro protonation constants obtained. It should also be noticed that mixed-cation species like $[K_x(H_yL)]^{(6-x-y)-}$ may be "hidden" under these effective constants.

With this in mind, we present a study of the inframolecular acid–base processes for the protonation of the Ins(1,2,3)P_3, over a wide pH range, analyzing the ^{31}P NMR curves by means of a model based on the "cluster expansion method" [13]. The experimental ^{31}P NMR data have been collected at 37.0 °C in the non-interacting medium 0.15 M NMe_4Cl which approaches physiological conditions. The complexation macroconstants of Ins(1,2,3)P_3 with K(I) have also been determined from these data. Although K(I) interaction with Ins(1,2,3)P_3 is not very strong, the fact that it is the major intracellular cation, means potassium species are no doubt relevant under physiological conditions.

Finally, we have performed a theoretical *ab-initio* approach to the energetic and structural analysis of the protonation and conformational changes of Ins(1,2,3)P_3 as the pH varies and how it is disturbed by the presence of K(I).

2. Experimental

2.1. Chemicals

All laboratory chemicals used throughout this work were reagent grade, purchased from commercial sources and used without further purification. KCl was used as metal source. All solutions were prepared with ultrapure water obtained from a Millipore-MilliQ plus system, the ionic strength being adjusted to 0.15 M with NMe_4Cl (Fluka), and used immediately after preparation. The tetrasodium salt of Ins(1,2,3)P_3, $Na_4H_2L{\cdot}2H_2O$, where L denotes the fully deprotonated form of the molecule, was prepared as previously reported [4]. Ins(1,2,3)P_3 solutions were prepared by weighing the appropriate amount of the salt. Standard HCl and NMe_4OH (Merck) solutions were prepared and standardised according to standard techniques.

2.2. ^{31}P NMR determinations

The chemical behaviour of Ins(1,2,3)P_3 was analyzed at 37.0 °C in 0.15 M NMe_4Cl. In order to do this, the ^{31}P NMR spectra of 5–10 mM Ins(1,2,3)P_3 solutions at different pH values (1.1, 2.0, 3.8, 5.4, 6.4, 7.4, 8.3, 9.4, 12.1) were recorded. The interaction of Ins(1,2,3)P_3 with K(I) was then studied under similar conditions. 2.5 - 5 mM Ins(1,2,3)P_3 solutions were prepared, with the necessary addition of the metal ion salt to give a final K(I) concentration of 0.5 M. pH was adjusted (1.2, 4.3, 5.9, 7.0, 7.8, 8.7, 10.0, 11.7) in order to have the predominance of a given species, by means of the information we have reported previously [4].

One-dimensional ^{31}P NMR spectra were recorded on a Bruker Avance 400 MHz instrument (161.98 MHz). All spectra were referenced externally to 85% H_3PO_4 (0.0 ppm) with downfield shifts represented by positive values. Field-frequency lock was achieved using D_2O insets with the aim of not altering the aqueous samples by the addition of D_2O. Spectra were recorded over a spectral width of 400 ppm using a 1 s relaxation delay. The temperature was controlled at 37.0 °C through the measurement of proton chemical shifts in a standard of 4% methanol in deuterated methanol. The phosphorus resonances were assigned according to the integration of the peaks.

2.3. Macro and micro acid–base studies

The ^{31}P NMR experimental data versus pH were analyzed using the HypNMR 2006 software [14]. This allowed us to determine the macroscopic protonation and the stability constants for every system in addition to the individual phosphorus chemical shifts for all species. In all cases, the fit of the values predicted by the model to the experimental data was estimated on the basis of the parameter σ, corresponding to the scaled sum of square differences between predicted and experimental values. With the aim of determining the microscopic protonation patterns over the studied pH range, the NMR data were analyzed by the "cluster expansion method" [13].

2.4. Calculations

All geometry optimizations in this report were performed using the modified GDIIS algorithm, in gas phase and by means of the methods described hereinafter as implemented in Gaussian 03, Revision B.01 package [15]. The final structures obtained were all minima in the potential energy surface, being the nature of the stationary points verified through vibrational analysis.

For the theoretical study on the Ins(1,2,3)P_3 protonation process, RHF geometry optimization runs were carried out using a 3-21+G* split valence basis set, which has suitable size to deal with this type of structures. This set contains diffuse functions added to carbon, oxygen and phosphorus atoms that allow orbitals to occupy a larger region of space and, in consequence, they are important for systems with significant negative charge and strong intramolecular hydrogen bonding. It also includes *d*-type polarization functions applied to P atoms, improving the description of the P–O bond. In order to obtain the initial inputs for L^{6-}, HL^{5-}, H_2L^{4-}, H_3L^{3-}, H_4L^{2-} and H_6L species in both conformations, 1 axial-5 equatorial (1a5e) and 5 axial-1 equatorial (5a1e) (Fig. 1), we started from the optimized fully deprotonated structure. Then, one proton was added at a time, according to the particular protonation pattern determined in this report. The geometries were then distorted to provide many starting conformational points, and all the systems were optimized. The minimum energy structure was selected for each species. On the basis of those optimized geometries, single point RB3LYP/3-21+G* calculations were run on them. In order to determine the energy of those RHF structures in

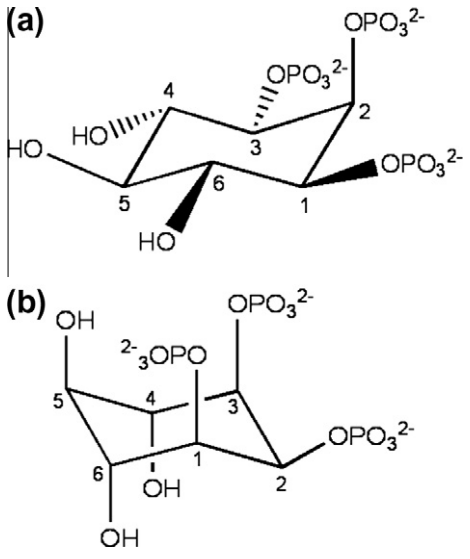

Fig. 1. Structure of Ins(1,2,3)P_3 (L^{6-}), for both conformations: (a) 1 axial-5 equatorial (1a5e) and (b) 5 axial-1 equatorial (5a1e).

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