EurJIC
European Journal of Inorganic Chemistry

A Journal of ChemPubSoc Europe

Accepted Article

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To be cited as: *Eur. J. Inorg. Chem.* 10.1002/ejic.201900091

Link to VoR: http://dx.doi.org/10.1002/ejic.201900091

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Structural Impact of Chelation on Phytate, a Highly Phosphorylated Biomolecule

Molly Reinmuth, Subhamay Pramanik, Justin T. Douglas, Victor W. Day, and Kristin Bowman-James*[a]

Abstract: An important biomolecule found in plant seeds and tissues, and in eukaryotic cells is *myo*-inositol-1,2,3,4,5,6-hexakisphosphate (phytate, IP_6). Phytate has many roles, including phosphate, *myo*-inositol, and mineral storage and retrieval in plants, and a number of metabolic roles, not all of which are known. Despite the importance of phytate in biology, structural information is limited. Aside from this report of the potassium phytate structure, $K_3[H_9IP_6]\cdot 2H_2O$, only the structures of the sodium and zinc salts have appeared. The potassium structure reveals the importance of metal ion chelation in stabilizing the conformation, and the two previously reported structures support this finding. Potassium ion and hydrogen bond bridges link the interwoven phytate networks throughout the lattice. ^{1}H NMR (800 MHz) titrations show the conformation crossover from the 1a5e to the 5a1e conformation between pH 9 and 10, and detailed ^{1}H deconvolution studies at low pH reveal the underlying pattern assignments for individual protons.

Introduction

Global concern over the depletion of available sources of phosphorus[1] has led to an increased awareness of the ubiquitous presence of inositol phosphates in the plant metabolic kingdom. Agricultural chemists and biologists long ago realized the important roles that inositol phosphates play in agriculture.[2] Within the inositol family, phytate, *myo*-inositol-1,2,3,4,5,6-hexakisphosphate (IP_6^{12-}), comprises from 20-50% of the organophosphates found in soils, and is one of the most studied of the inositol phosphates.[3-6]

Phytate has been found to have numerous physiological functions. It plays a major role as a vehicle for mineral storage and retrieval in plant seeds and grains,[3] and is involved in plant metabolism, signal transduction, and cell regulation.[4] Because of its highly charged nature at physiological pH ($H_4IP_6^{8-}$ and $H_5IP_6^{7-}$),[7] its ability to readily trap metal ions has resulted in its classification as an anti-nutrient. Yet, despite its notoriety, some of phytate's functions are still unknown, and a better understanding of its chemistry has been limited due to the lack of crystallographic structural information.

K^+ ion is one of the crucial metal ions in seed development and is stored in seeds as the phytate salt.[3] Hence, structural information about interactions of K^+ ion with phytate is especially important. Even at a lower pHs, structural findings can help to understand modes of binding, e.g., chelating, bridging, or combinations of both, which influence the structure and chemistry of this important biological anion.

The *myo*-inositol stereoisomer occurs primarily in two forms depending on pH. The 1a5e phosphate conformation at lower pHs switches to the 5a1e form between pH 9 and 10 (Figure 1). While phytate impacts many fields of science due to its large phosphorus content (28.42% in phytic acid), only two crystal structures of metal ion salts have been reported. In the Na^+ structure, $[Na]_{12}[IP_6]\cdot 38H_2O$, reported in 1971,[8] the IP_6^{12-} is in the 5a1e conformation, while in the Zn^{2+} structure, $[Zn]_{10}[H_2IP_6]_2\cdot 14H_2O$, reported in 2017, the $H_2IP_6^{10-}$ is in the 1a5e conformation.[9] More recently, Kremer and Bianchi have used both supramolecular and transition metal coordination chemistry to crystallize a handful of transition metal and supramolecular complexes of phytate at more intermediate pH ranges.[10-12] However, structural information about simple metal salts of phytate is still woefully lacking.

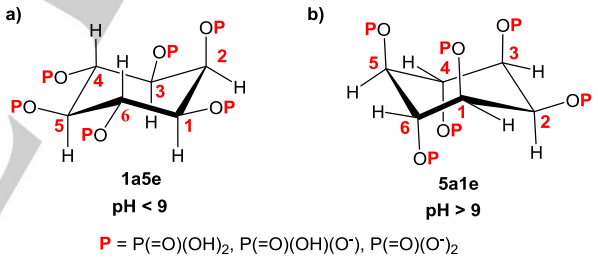

Figure 1. The two primary conformations of phytate, **(a)** 1a5e and **(b)** 5a1e.

Here we report the crystal structure of the K^+ salt, $[K]_3[H_9IP_6]\cdot 2H_2O$, which crystallizes in the 1a5e conformation. Crystals were grown from the dipotassium salt of phytic acid, $[K]_2[H_{10}IP_6]$, obtained from Sigma Aldrich. The salt was dissolved in 0.5 mL of water. After sitting at room temperature for one week, 0.5 mL of water was added to give a slight yellow color and a pH of 1.2. Filtration of the solution was followed by vapor diffusion with acetone for two weeks. Crystals suitable for X-ray analysis were isolated on additional slow evaporation to dryness. Unfortunately, attempts to grow K^+ crystals at higher pHs have so far been unsuccessful.

The K^+ phytate salt crystallized in the monoclinic space group $P2_1/c$, with three crystallographically independent K^+ ions and two water molecules of crystallization, $[K]_3[H_9IP_6]\cdot 2H_2O$ (Figure 2(a)). The phytate ion is in the 1a5e conformation surrounded by 10 K^+ ions within a 3.00 Å distance. Most of the phosphate hydrogen atoms were located from a difference Fourier, and their assignments correlated well with the refined P-O bond lengths. However, the location of the negatively charged phosphates was somewhat problematic, complicated by a

[a] M. Reinmuth, Dr. S. Pramanik, Dr. J. T. Douglas, Dr. V. W. Day, Prof. K. Bowman-James
Department of Chemistry, University of Kansas
1251 Wescoe Hall Drive, Lawrence, KS 66045 (USA)
E-mail: kbjames@ku.edu
Homepage: https://chem.ku.edu/people/faculty/bowman-james

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hydrogen atom (O62H) sitting on an inversion center, and shared between two phytates. After careful examination of P-O bond distances, the most reasonable assignment appears to be a dinegative charge on P5, which shares a very short hydrogen bond (2.494(6) Å) with P6. The third mononegative charge is on P4.

The most significant structural finding is the prevalence of metal ion chelation not just for K^+ phytate, but now in all three reported structures (Figure 2). K^+ chelate associations are observed between each pair of adjacent phosphates with the exception of P5 and P6 (Figure 2(a)). Five bidentate and one tridentate chelates are observed, the latter being K_2* with P_2, P_3, and P_4. K^+-O distances range from 2.7 to 3.1 Å. Two K^+ ions (K1 and K2*) and a bridging O1w water molecule connect the axial P2 and equatorial P3. The single intramolecular hydrogen bond in the molecule is quite short (O63-H···O54 = 2.494(6) Å) and is between the only two phosphates without a K^+ bridge (Figure 2(a)).

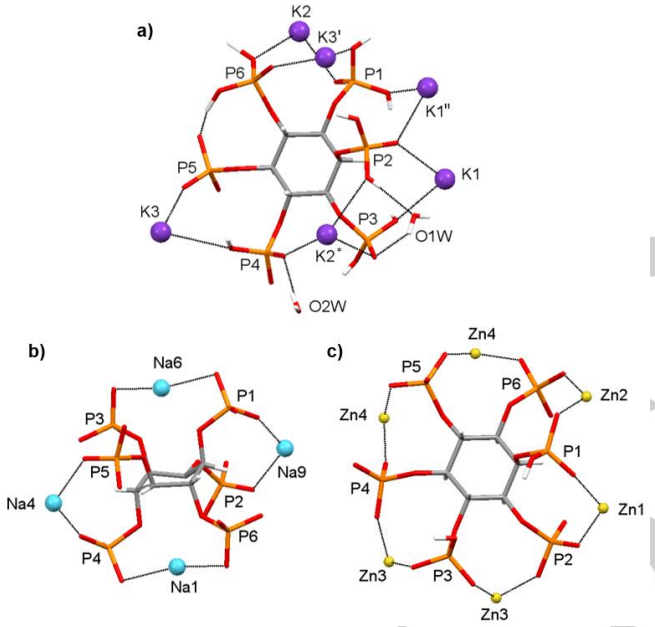

Figure 2. (a) Overhead view of $[K]_3[H_9IP_6]\cdot 2H_2O$ showing six K^+ chelate interactions. Symmetry relations: ' = -x, -y+1,-z+1; " = -x+1, -y, -z+1; and * = -x+1, -y+1, -z+1. **(b)** Side view of $[Na]_{12}[IP_6]\cdot 38H_2O$ showing four Na^+ chelates.[8] **(c)** Overhead view of $[Zn]_{10}[H_2IP_6]$ showing six Zn^{2+} chelates. Atom labelling schemes for **(b)** and **(c)** are from the reported cif files.[8-9] Only chelating metal ions are shown.

Structural influences of metal ions with different charges and sizes, and of phytate with different charges and conformations can now be compared given that crystallographic data are available for three different metal ion phytate salts.[8-9] The Na^+ salt structure provides a picture of phytate in the high pH 5a1e conformation.[8] The Zn^{2+} structure illustrates the influence of higher charge and smaller metal ion size on phytate chelation, and provides additional insight to the stabilization of the 1a5e conformation at higher pHs.[9]

In the Na^+ structure, the −12-charged phytate ion chelates to four Na^+ ions, with Na^+-O distances averaging 2.39 Å (Figure 2(b)). More importantly the Na^+ ions link axial phosphates across the top (Na6 to P1 and P3) and bottom (Na1 to P4 and P6) of the phytate. O---O distances between the two phosphate pairs are 4.80 and 4.85 Å. Hence, these two Na^+ ions assist in locking the 5a1e conformation in place by tying together four of the axial phytates. As Sax and coworkers point out, these linkages help to stabilize the "otherwise sterically undesirable conformation."[8]

The Zn^{2+} salt was synthesized by Zhu and coworkers to provide a metal-organic framework (MOF) for use in a polymer composite membrane for proton exchange.[9] In spite of the highly charged nature of the Zn^{2+} structure (−10), the salt crystallizes in the 1a5e conformation (Figure 2(c)). There are two crystallographically independent, pseudo-centrosymmetric phytate anions. Only one is described due to their almost identical structures. P1, the axial phosphate, and P3 are protonated. Each pair of phosphates is bridged by a Zn^{2+} ion. Zn-O distances range from 1.83 to 1.96 Å, and are the shortest of the three metal ions. The resulting short O---O distances through the chelating Zn^{2+} ions (average 3.22 Å) undoubtedly help to stabilize the 1a5e conformation past the normal pH of 9 – 10 observed for conversion to the 5a1e form. These findings support Veiga and co-workers' ^{31}P studies for divalent Mg^{2+} and Ca^{2+} salts,[13] in which the 1a5e conformation appears to be the most stable conformation for $H_2IP_6^{10-}$. The Zn^{2+} MOF was also found to be highly stable in the presence of acidic environments (pHs 2 - 5), which was attributed to the very tight Zn-OP connections.[9]

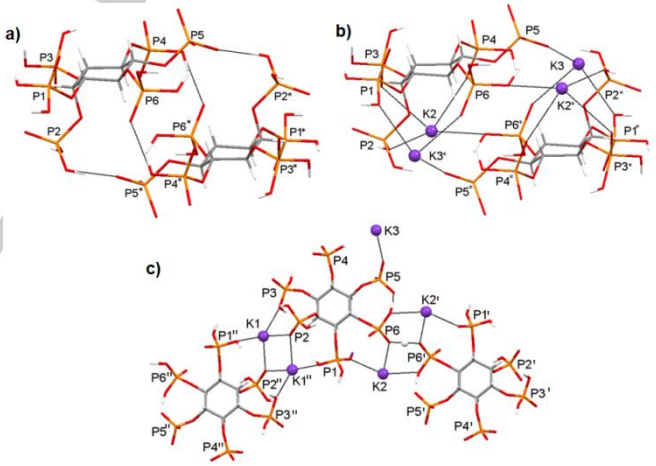

Figure 3. Views of **(a)** the hydrogen bond and **(b)** the K^+ ion interactions in the nestled ion pair, and **(c)** the three in-plane phytate associations. Symmetry relations: ' = -x, -y+1,-z+1; " = -x+1, -y,-z+1; and * = -x+1, -y+1, -z+1.

The crystal lattice for the K^+ salt reveals extended and intricate K^+ salt networks interwoven with strong interionic hydrogen bonds (Figure 3). Each phytate interacts with neighboring phytates via intermolecular hydrogen bonds and potassium bridges. Three interactions in particular are of note. A close-knit, interlocked dimer pair results from strong intermolecular hydrogen bonds and K^+ bridges (Figure 3(a) and (b)). Two other nearly co-planar, neighboring interactions are

observed with H_9P' and H_9P'' on either side of the central phytate (Figure 3(c)), also with K^+ ion bridges.

The close-knit dimer pair interactions consist of four intermolecular hydrogen bonds from two symmetry-related pairs of phosphates, one between oxygen atoms on P2, P5* and P5, P2* (2.431(6) Å) and the second between P4, P6* and P6, P4* (2.574(10) Å) (Figure 3(a)). Two pairs of symmetry-related K^+ ions, K2,K3 and K2*,K3*, also serve to pull the two phytate ions together (Figure 3(b)). K2 joins oxygen atoms on P2, P3, and P4 with P6* oxygen atoms of the phytate below and vice-versa with K^+-O distances ranging from 2.80 to 2.98 Å. K3 ions join oxygen atoms on P1* and P6* with an oxygen on P5 and vice-versa (2.695(5) and 2.732(5) Å).

The inter-phytate interactions with the two coplanar phytates primarily involve K^+ interactions, with the exception of one very strong hydrogen bond between P6 and P6' (O62···H^+···O62' = 2.435(9) Å). The tie between the two inversion-related phytates is strengthened by the presence of two K^+ bridges (K2 and K2') (Figure 3(c)). No direct hydrogen bonds are observed between the central phytate and its neighbor to the left. Rather, K1 and K1" ions form a diamond-like pattern joining the two phytates through three symmetry-related oxygen atoms on P1, P2, and P3 and with the corollary H_9P'' groups (Figure S1). There are four additional intermolecular hydrogen bond interactions to nearby phytates, involving protonated P1 and P3 oxygen atoms, which will be described in greater detail in a later paper.

NMR spectroscopy has been an invaluable tool for studying phytate, especially considering the multinuclear and multi-dimensional NMR tools currently available. In the absence of crystallographic data, it has been an incredible asset in pinpointing conformational changes and sequential phosphate protonation sequences in phytate as a function of pH and different metal ions.[13-15] Previous focus has been on ^{31}P and ^{13}C spectra, including 2D and deconvolution studies.[15] While ^1H NMR is also a viable probe, it tends to be very sensitive not only to changes in protonation of the phosphates, but also to intramolecular proton migration as a function of both pH and metal ion binding.[13] Here we probe the ^1H NMR (800 MHz) as a function of pH for the K^+ salt. Initial spectra were measured at the approximate pH of the isolated crystals, and the pH was raised through addition of aliquots of KOH to a final pH of 12.95. ^{31}P and ^{13}C spectra are included in the Supporting Information (Figures S16 and S12, respectively).

The pH-profile of the ^1H NMR spectra (800 MHz) indicates the dramatic chemical shift changes on conversion between the two phytate conformations (Figure 4(a) and S17). The initial spectrum of the phytate salt at pH 1.68 shows the anticipated signals (filled circles) corresponding to the least hindered, deshielded H2 (dt), downfield at 5.0 ppm, and overlapping axial H4/H6 (q) and H1/H3/H5 (m) protons, upfield between 4.6 and 4.35 ppm (Figures 4(a) and S2). Tiny signals seen upfield are due to small amounts of other inositol phosphate impurities that usually accompany phytate salts obtained commercially.

Deconvolution patterns obtained at pH 1.68 for the H4/H6 and H1/H3/H5 signals clearly show the anticipated underlying patterns due to coupling with vicinal hydrogen and phosphorus nuclei (Figure 4(b)). Coupling constants were about 2.4 Hz for axial/equatorial proton couplings [$^3J(H_{ax}$-$H_{eq})$] and 9.4-9.6 Hz for diaxial [$^3J(H_{ax}$-$H_{ax})$] and [^3J(H-P)] couplings (Figures S8-S9, Tables S4-S5). For the 1a5e conformation (pHs < 9) equatorial H2 couples with the axial P2 and then with axial H1/H3 for a doublet of triplets (dt). Axial H1/H3 protons couple with equatorial P1/P3 and axial H4/H6 followed by the equatorial H2 for a triplet of doublets (td). The axial protons H4/H6 and H5 are coupled with vicinal axial protons and the corresponding phosphorus atoms to generate a quartet (q) (Figure 4(b)).

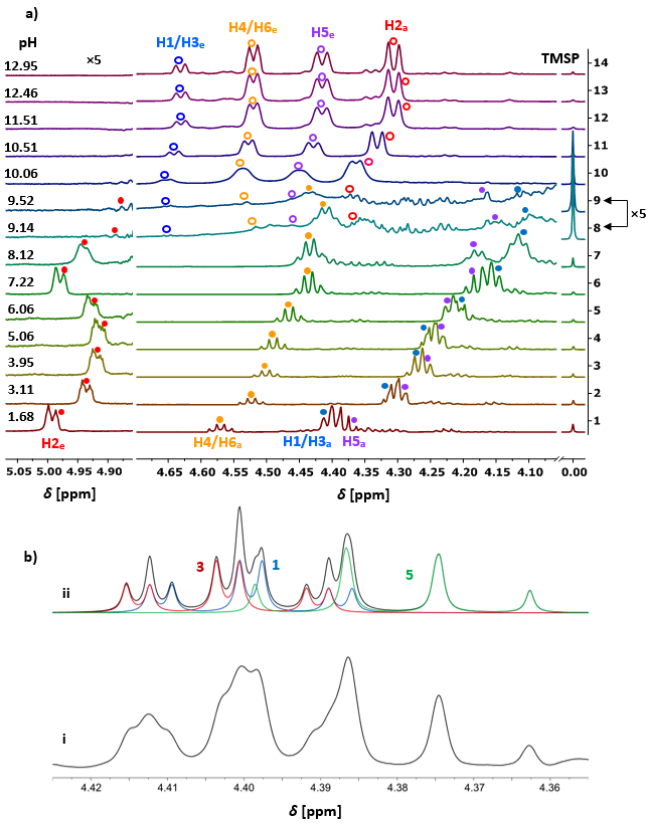

Figure 4. (a) ^1H NMR spectra (with water suppression, 800 MHz) of the phytate salt at pH 1.68 as a function of pH (D_2O, 25°C) using trimethylsilylpropanoic acid (TMSP) as an internal reference. Signals due to 1a5e and 5a1e configurations are marked by filled and unfilled circles, respectively; **(b)** (i) Stack plot of the region around 4.39 ppm recorded at pH 1.68 and (ii) simulated spectrum based on parameters listed in Tables S4 and S5. Deconvolution of the individual components are included in the simulated (ii) with resonance signals 1, 3, 5, and summation in blue, red, green, and black, respectively.

^1H NMR titrations are complicated because multiple species exist at any pH. Furthermore, protonation shields while metal ion binding deshields phytate hydrogens, so chemical shifts are the result of a delicate balance between the two influences.[13] As the pH increases with addition of KOH, the axial proton signals shift upfield until pH 9.14, due to increased electron density on the phosphates from deprotonation (Figure 4(a)). The equatorial H2 only shifts slightly upfield. At pH 6.06 H2 starts back downfield, possibly the result of rapid H^+ and K^+ exchange between phosphates, and H1/H3 hydrogens become the most upfield-shifted signals. At pH 8.12, the signals start to broaden, and at pH 9.52 the 1a5e signals almost disappear as

new signals appear in the 4.30 to 4.65 ppm region. At pH 10.06 four signals remain, H1/H3, H2/H4, H5, and H2, each a doublet of triplets (dt), indicative of the 5a1e conformation. The sharpness of the conformation transition is captured in a plot of chemical shifts as a function of pH (Figure 5). Potentiometric studies by Veiga and coworkers indicate potassium phytate is transitioning from $[K_5(H_2IP_6)]^{5-}$ (1a5e) to $[K_6(IP_6)]^{6-}$ species approaching pH 9.5.[13]

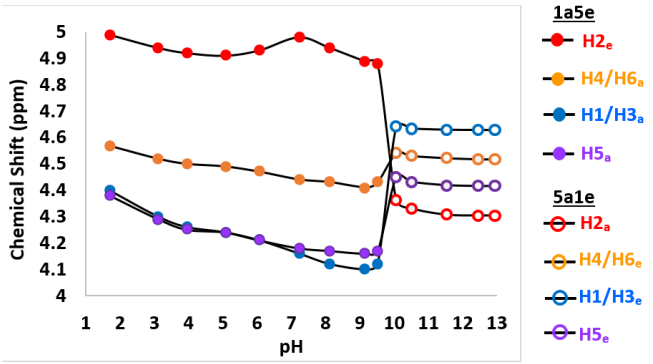

Figure 5. Variation in the chemical shift of proton signals of the K^+ salt of phytate as a function of pH.

Conclusions

In summary, this is the first structural report of a K^+ phytate salt. By comparing the K^+ structure with the two other metal ion salt structures, charge and chelation effects on phytate conformations can now be assessed. The K^+ structure has a complex chelate network involving six K^+ ions, and interionic K^+ bridges play a major role in the formation of a tight-knit dimer pair. In the Na^+ salt, Na^+ chelation across two phosphates above and below the phytate clearly stabilizes the 5a1e conformation, while in the Zn^{2+} salt, a tight Zn^{2+} chelate girdle around the 1a5e conformation locks it tightly in place. Intramolecular hydrogen bonding does not appear to play a major role in the K^+ structure, although intermolecular hydrogen bonding is observed. The high field ^1H NMR studies, along with proton deconvolution studies, provide more highly resolved titration curves for the conformation change between 9 and 10 as well as confirmation of the underlying pattern assignments for the overlapping signals at low pH.

In conclusion, our results, along with those of the Na^+ and Zn^{2+} structures,[8-9] provide insight to the role metal ion chelation plays in the stabilization of both the 1a5e as well as the 5a1e conformations of phytate. Additionally, the influence of both size and charge on chelation modes may, in part, be responsible for variations in the pH cross-over points between phytate conformations observed for different metal ions. This valuable structural insight will help to enable a better understanding of the metal ion chemistry not only of phytate, but also of other complex biomolecules and ions.

Acknowledgments

The authors thank the National Science Foundation (CHE-1710535) for support of this work and (CHE-0923449) for the purchase of the X-ray diffractometer. Support for the NMR instrumentation was provided by an NIH Shared Instrumentation Grant #S10OD016360 and COBRE center grant P30 GM110761. The authors also thank Professor Carlos Kremer, for helpful discussion.

Keywords: potassium phytate • structure elucidation • X-ray diffraction • NMR spectroscopy • inositol phosphates

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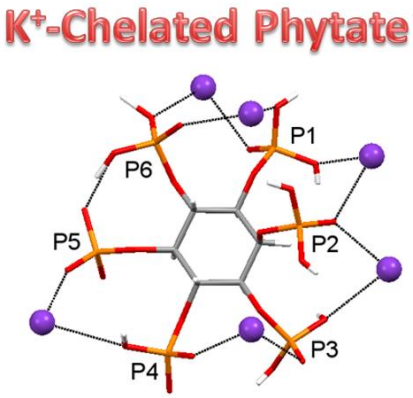
Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

The crystal structure of the potassium salt of phosphorylated *myo*-inositol (phytate), a multi-tasking biological anion, provides insight to the structural influence of metal ion chelation. Potassium ions and hydrogen bond bridges also serve to interlink phytate ions throughout the crystal lattice. High field proton NMR studies provide solution snapshots of conformation changes as a function of pH.



Key Topic: Phytate Chelates

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