Conformational change of metal phytates: Solid state 1D $^{13}$C and 2D $^1$H-$^{13}$C NMR spectroscopic investigations

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Abstract

Phytate is an important phosphorus and mineral storage compound in plant seeds. Both the benign and adverse effects of phytate in nutrition and environment are mainly due to its unique conformational structures, where a strong chelating ability makes phytate interact with many cations (such as Zn$^{2+}$, Ca$^{2+}$, Mg$^{2+}$, and Al$^{3+}$). However, information is scant on the conformational forms of different solid metal phytate compounds although phytate in solution exists in two conformations: one axial and five equatorial phosphates (1a/5e structure) and an inverted 5a/1e structure. Consequently, we investigated the spectral features of nine representative metal phytate compounds by solid state 1D $^{13}$C cross polarization magic angle spinning (CPMAS) and 2D $^{13}$C-$^1$H heteronuclear correlation (HETCOR) NMR. A broad peak appeared in all solid 1D NMR spectra of hydrogen monovalent, divalent, and trivalent metal phytate compounds. The spectra of hydrogen monovalent and divalent compounds could be deconvoluted to two separate resonance peaks. 2D HETCOR clearly showed distinct $^{13}$C-$^1$H correlations for inositol C-H moieties in hydrogen metal phytates from non-hydrogen metal phytates. Through spectral comparison, this work demonstrated that different valent cations in solid phytate compounds could induce conformational changes of the inositol ring as pH does on the phytate in solution. Therefore, the current knowledge on the effect of pH on phytate conformation can be helpful in understanding the conformational changes of phytate when it interacts with different valent metals to form less soluble even insoluble compounds in the environment.

Key words: Inositol phosphate, phytate, NMR, CPMAS, HETCOR, conformational change.

Introduction

Phytate, the salt of phytic acid, is the common name of myo-inositol-hexakis (dihydrogen phosphate) (IP$_6$), or 1,2,3,4,5,6-cyclohexanhexolphosphoric acid. It is an important phosphorus (P) and mineral storage compound in plant seeds, such as cottonseed $^1$. It is estimated that over 51 million metric tons of phytate (in K and Mg salts) are produced in crop seeds and fruits globally each year $^2$. Phytate is often considered an antinutrient substance that has an adverse effect on human and animal nutrition $^3$. Soybean meal and cottonseed meal in poultry feed contain about 2.2 and 4.4% (w/w) of phytate, respectively. However, up to two-thirds of P bound to phytate in these meals are unavailable to poultry $^4$. Phytate reduction has been used in bread making to improve the quality and nutrient values of bread $^5$. On the other hand, phytate has been reported to be a potential detoxicating and anti-cancer agent $^6,7$. In regards to environmental concern, phytate represents a significant portion of P in animal manure, and its runoff and leaching play a role in eutrophication of surface waters $^8,9$.

Both the benign and adverse effects of phytate in nutrition and environment are mainly due to its unique structure, where strong chelating ability makes phytate interact with many cations (such as Zn$^{2+}$, Ca$^{2+}$, Mg$^{2+}$, Cu$^{2+}$, Mn$^{2+}$, and Fe$^{3+}$, Al$^{3+}$) $^{10-13}$. Structurally, phytate contains a 6-carbon ring with 1 hydrogen and 1 phosphate attached to each carbon. Each of the 6 phosphate groups is attached in an ester linkage and retains 2 hydrolyzable hydrogens. Generally, there are two possible conformations for a cyclohexyl ring: a chair or a boat structure. Previous studies confirm that phytate is present in the chair form. Solution $^{13}$P and $^1$H NMR studies $^{14,15}$ have shown that above pH 9.5 five phosphate groups attach to the inositol ring in the axial position and one phosphate group in the equatorial position (5a/1e conformation). Below pH 9.2, phytate assumes a 5e/1a conformation (Fig. 1). Based on the $^1$H and $^{13}$C NMR spectral data, Paton et al. $^{15}$ proposed that the structural change is triggered by the ninth acid dissociation of phytic acid, and the stabilization of the axial and equatorial conformations is due partly to the presence of C-H…O-P through-space interactions, and trans-annular hydrogen bonding between all the phosphate groups. Nevertheless, the information on the conformational status of solid (insoluble) metal phytate compounds is very limited although such information could be useful in understanding the roles of phytate in metal storage in plants $^{16}$, lability and bioavailability of organic P compounds in the environment $^{17,18}$, and immobilization of heavy metals in contaminated soils $^{19}$.

In the limited literature available, an X-ray analysis revealed the 5a/1e conformation of sodium phytate $^{20}$. In contrast, solid (hydrated) Ca$_4$IP$_6$ was characterized as 1a/5e conformer by Raman
and Fourier Transform Infrared (FTIR) spectroscopic analysis\textsuperscript{21}. To model metal storage compounds in wheat grains, solid monometallic and heterometallic Mn/Zn phytate complexes were characterized with high resolution\textsuperscript{22}Na,\textsuperscript{13}C, and\textsuperscript{31}P NMR, electron paramagnetic resonance (EPR) and X-ray photoelectron spectroscopy (XPS)\textsuperscript{16}. The results suggested different conformations among the three model phytate complexes of transition metals. He \textit{et al.}\textsuperscript{11,12,22} comparatively characterized a series of solid metal phytates and corresponding orthophosphates by FTIR,\textsuperscript{18}P NMR, and X-ray absorption near edge structure (XANES) spectroscopy, demonstrating that these solid state spectroscopic techniques could be applied to metal speciation of phytate in environmental and other samples. Based on FTIR data, possible metal-coordinate bonding conformations of phytate compounds with mono-, di-, and trivalent metals were presented\textsuperscript{12}. However, the aforementioned spectroscopic research\textsuperscript{11,12,22} did not answer the question if there are any conformation changes of the 6-C ring among these metal phytate compounds.

In this study, we report solid state 1D\textsuperscript{13}C and 2D 1H-1\textsuperscript{3}C NMR spectra of 9 metal phytate compounds. Spectral analysis provides evidence for conformational changes that are influenced by pH, temperature, and different valent cations.

**Materials and Methods**

**Materials:** myo-Inositol, dodecasodium phytate (Na\textsubscript{12}IP\textsubscript{6}), potassium/magnesium hydrogen phytates (K\textsubscript{2}H\textsubscript{10}IP\textsubscript{6}, KMgH\textsubscript{9}IP\textsubscript{6}, and K\textsubscript{2}Mg\textsubscript{2}IP\textsubscript{6}), and K\textsubscript{2}Mg\textsubscript{2}IP\textsubscript{6}. were purchased from Sigma (St. Louis, Missouri). Divalent (Ca, Mg, Zn, and Cd) and trivalent (Al) phytates were prepared in house and reported previously\textsuperscript{11,12}. All materials were kept in a desiccator at room temperature until use.

**1D \textsuperscript{13}C NMR measurement:** 1D solid state NMR experiments were performed on a Bruker AVANCE II 400 MHz NMR spectrometer (Bruker-Biospin, Billerica, MA) with 13C frequency at 100.61 MHz equipped with a 4-mm double-resonance Magic Angle Spinning (MAS) probe. Myo-Inositol and other phytate compounds were directly used for solid NMR analysis. The 1D Cross-Polarization MAS (CPMAS) spectra were recorded with 1.5 ms contact time, 4 μs\textsuperscript{13}C pulse length, 1 s recycle delay and two-pulse phase modulation (TPPM) proton decoupling during acquisition.

**2D 1H-\textsuperscript{13}C NMR measurement:** The 2D solid state NMR spectra were recorded in the same Bruker AVANCE II spectrometer. Approximately 60 mg solid sample was introduced to the 4-mm zirconia rotor and sealed with a Kel-F cap. The spinning speed was kept at 12±1 kHz. The 2D Heteronuclear Correlation (HETCOR) pulse sequence was used, where a proton RF field of 62.5 kHz was applied for the two-pulse phase modulation (TPPM) proton decoupling during acquisition\textsuperscript{23}. The mixing time and the 1\textsuperscript{3}C pulse length were 10 ms and 4 μs, respectively. The chemical shifts were calibrated externally with \textsuperscript{13}C labeled glycine. The data were processed with the Topspin software version 2.1 (Bruker-Biospin, Billerica, MA). Spectral analysis was performed using SpinWorks version 3.1\textsuperscript{24}.

**Results and Discussion**

Solid state 1D\textsuperscript{13}C NMR spectra of myo-inositol and metal phytates CPMAS is a widely used solid state\textsuperscript{13}C NMR technique that improves sensitivity with the transfer of magnetization from the \textsuperscript{1}H to \textsuperscript{13}C spins through cross-polarization\textsuperscript{25}. The\textsuperscript{13}C CPMAS NMR spectrum of myo-inositol features three peaks at 74.5, 72.3 and 70.5 ppm (Fig. 2). Deconvolution analysis indicated the peak intensity ratio of the solid state NMR peaks of myo-inositol to be 1:3:2 (Table 1). Previously, proton-decoupled liquid\textsuperscript{13}C NMR spectroscopy of aqueous solution of myo-inositol revealed 4 peaks at 75.5, 73.5, 73.2 and 72.2 ppm with the 1:2:1:2 pattern of relative integrated intensities\textsuperscript{21}. It is apparent that the two middle peaks in the liquid NMR spectrum appear as a single peak in the solid NMR spectrum whereas all the chemical shifts moved downfield in the solid state NMR spectrum. This difference can be explained by the anisotropic interaction in the solid state that leads to broad CPMAS spectra\textsuperscript{25}. The crystal structure of solid myo-inositol is in the 1\textsubscript{a}/\textsubscript{5e} conformation\textsuperscript{26}. The myo-inositol in aqueous solution was assumed to keep this conformation so that the four NMR peaks at 75.5, 73.5, 73.2 and 72.2 ppm was assigned to C5, C1/3, C2, and C4/6 in that order\textsuperscript{21}. With this literature information, we can assign the three solid state\textsuperscript{13}C NMR spectral peaks of myo-inositol at 74.5, 72.3, and 70.5 ppm to C5, C2/1/3, and C4/6, respectively.

The\textsuperscript{13}C CPMAS NMR spectrum of Na\textsubscript{12}IP\textsubscript{6} shows a major peak at 72.9 ppm and a minor peak at 66.3 ppm with the peak intensity ratio of 5:1 (Fig. 2 and Table 1). This observation is consistent with a previous report\textsuperscript{16} that showed the\textsuperscript{13}C CPMAS NMR spectrum of Na\textsubscript{12}IP\textsubscript{6} consisting of two partially resolved peaks at 74 and 67 ppm with a relative intensity ratio of 5:1. X-ray analysis revealed the 5a/1e conformation of solid sodium phytate\textsuperscript{20}. Based on the intensity ratio, it is reasonable to assign the peak at 66.3 ppm to the C2 with the only axial phosphate group\textsuperscript{16}. We accordingly assign the two solid state\textsuperscript{13}C NMR spectral peaks of Na\textsubscript{12}IP\textsubscript{6} in this study to C5 and C1/2/3/4/6 in the 5a/1e conformation. It should be noted that this solid spectral feature and assignment of Na\textsubscript{12}IP\textsubscript{6} is similar but not identical to those of aqueous sodium phytate solution at high pH. At pH>10.0, the solution\textsuperscript{13}C NMR spectra of 0.10 M aqueous sodium phytate solution show three peaks at 74.5, 71.5, and 67.5 ppm with the intensity ratio of 4:1:1. The three peaks were assigned to C1/3/4/6, C2, and C5, respectively\textsuperscript{21}.

**Solid state 1D \textsuperscript{13}C NMR spectra of other metal phytates:** The spectra of Al\textsubscript{12}IP\textsubscript{6}, Ca\textsubscript{12}IP\textsubscript{6}, Cd\textsubscript{12}IP\textsubscript{6}, and Mg\textsubscript{12}IP\textsubscript{6} look like a broad single peak between 73-74 ppm. Attempts have been made to deconvolute the peaks into two component peaks; however, the major component is clearly the large peak at ca. 73 ppm, and the second component is relatively low in intensity. Compared to

**Figure 1.** Two conformations of phytate compounds. For clarity, six phosphate (OPO\textsubscript{x}) groups attached to the inositol ring are simplified and shown as OP, where P= -PO\textsubscript{3}H\textsubscript{2}, -PO\textsubscript{3}H\textsuperscript{-} or –PO\textsubscript{3}\textsuperscript{2-} depending on compound. Note the label 1a (axial) or 1e (equatorial) refers to the axial or equatorial position of phosphate group associated with C2 (P2). Other five phosphate groups (5a or 5e) are in positions opposite to P2.
those of myo-inositol, the NMR peaks of Al$_4$IP$_6$, Ca$_6$IP$_6$, Cd$_6$IP$_6$, and Mg$_6$IP$_6$ have all moved downfield, similar to those of aqueous sodium phytate at lower pH. As in the case of Na$_{12}$IP$_6$, the large peak at ca. 73 ppm is characteristic of the 5a/1e conformation. Thus, we consider the conformation of these four compounds is essentially 5a/1e, perhaps with some distortion due to complexation with the metal ion.

### Figure 2.
Solid state $^{13}$C cross polarization magic angle spinning (CPMAS) NMR spectra of myo-inositol and 9 metal phytates. Heavy curves are original spectra; Light curves are deconvoluted spectral components with the peak shifts (ppm) labeled.

In the spectra of K$_2$H$_{10}$IP$_6$ and KMgH$_9$IP$_6$, two peaks at ca. 75 and ca. 72 ppm partly overlap, producing broad saddle-shaped or flattened peaks. Deconvolution analysis reveals that the intensity ratio of the two peaks is about 4:2. By analogy to myo-inositol spectrum, the peak at 75 ppm corresponds to C1/2/3/5, and the peak at 72 ppm corresponds to C4/6, and the conformation is mostly 5a/1e. The other two metal hydrogen phytates (K$_4$Mg$_2$H$_4$IP$_6$...
and Zn$_2$IP$_6$) possess somewhat similar spectral feature, but the separation between the two peaks is not as pronounced (Fig. 2). Deconvolution analysis gives ca. 3:3 as the intensity ratio for the separation between the two peaks is not as pronounced (Fig. 2).

### Table 1. Solid-state 1D $^{13}$C CPMAS NMR spectral features and deconvoluted components of myo-inositol and phytate (IP$_6$) compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Deconvoluted peaks ppm (relative intensity)</th>
<th>Peak intensity ratio$^1$</th>
<th>Possible Conformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myo-Inositol</td>
<td>74.5 (22.3%), 72.3 (49.5%), 70.5 (28.2%)</td>
<td>(1:3):2:0</td>
<td>1a/5e</td>
</tr>
<tr>
<td>Al$_4$IP$_6$</td>
<td>78.0 (21.0%), 73.8 (79.0%)</td>
<td>1:5:0</td>
<td>5a/1e, mostly</td>
</tr>
<tr>
<td>Ca$_4$IP$_6$</td>
<td>79.2 (11.9%), 73.7 (88.1%)</td>
<td>1:5:0</td>
<td>5a/1e, mostly</td>
</tr>
<tr>
<td>Mg$_4$IP$_6$</td>
<td>77.9 (19.1%), 73.8 (80.9%)</td>
<td>1:5:0</td>
<td>5a/1e, mostly</td>
</tr>
<tr>
<td>K$_4$H$_2$IP$_6$</td>
<td>75.3 (60.9%), 72.2 (39.1%)</td>
<td>4:2:0</td>
<td>1a/5e, mostly</td>
</tr>
<tr>
<td>KMg$_4$IP$_6$</td>
<td>75.2 (63.2%), 72.2 (36.8%)</td>
<td>4:2:0</td>
<td>1a/5e, mostly</td>
</tr>
<tr>
<td>K$_2$Mg$_4$IP$_6$</td>
<td>76.1 (45.5%), 72.9 (54.5%)</td>
<td>3:3:0</td>
<td>1a/5e + 5a/1e, mixed</td>
</tr>
<tr>
<td>Zn$_2$H$_2$IP$_6$</td>
<td>76.0 (43.3%), 73.5 (56.7%)</td>
<td>3:3:0</td>
<td>1a/5e + 5a/1e, mixed</td>
</tr>
<tr>
<td>Cd$_4$IP$_6$</td>
<td>~0:6:0</td>
<td>5a/1e</td>
<td>Mostly</td>
</tr>
<tr>
<td>Na$_2$IP$_6$</td>
<td>72.9 (84.5%), 66.3 (15.5%)</td>
<td>0:5:1</td>
<td>5a/1e</td>
</tr>
</tbody>
</table>

$^1$ Based on comparison of the observed peak intensity ratios and the theoretic percentages of 1 to 5 carbons in the six carbons of IP$_6$ (16.7%, 33.3%, 50.0%, 66.7%, and 83.3%).

In view of present data, the solid state NMR spectra of K$_4$Mg$_2$H$_4$IP$_6$ and Zn$_2$H$_2$IP$_6$ are due to either overlapping 1a/5e + 5a/1e conformations, or interconverting 5a/1e and 1a/5e conformations. In solution, sodium phytate showed two partially overlapped peaks at around 75 and 74 ppm and the spectral feature was interpreted as the average of the spectra of the two rapidly inter-converting 1a/5e and 1e/5a chair conformations. This interpretation may be applicable to the observations in this study. Depending on the relative amount of 1e/5a and 1a/5e conformations and the coalescence temperature of interconversion, different lineshapes are observed for the spectra of different phytate compounds. Since the metal is known to stabilize the 1e/5a structures, the identity of the metal has an effect on the lineshape as well. Some distortions from the 1e/5a and 1a/5e conformations are also possible, as pointed out by several authors.

**Solid state $^1$H-$^{13}$C HETCOR spectra:** 2D $^1$H-$^{13}$C HETCOR NMR is good for detecting connectivities of directly bound protons and carbons. The 2D $^1$H-$^{13}$C spectra of the four metal hydrogen phytates show separated or partially separated contour peaks. These results are consistent with 1D data, indicating that K$_2$H$_2$IP$_6$ and KMg$_4$IP$_6$ are mostly in the 1a/5e conformation with 10 ms mixing time.

[Figure 3. 2D $^{13}$C-$^1$H heteronuclear correlation (HETCOR) spectra of metal hydrogen phytates with 10 ms mixing time.]
1a/5e conformation, and K4Mg2H4IP6 and Zn5H2IP6 in mixed 1a/5e + 5a/1e conformations.

Whereas no 2D 1H-13C NMR spectra of phytate has been previously published, the 1H shifts of the 2D spectra in this work are smaller than the 1H shifts in the solution spectra of inositol phytates \(^{14,15}\). Compared to the 2D spectra of non-hydrogen metal phytate (Fig. 4), the upfield 1H contour peak in Fig. 3 could be attributed to protons at positions 1, 3 and 5 (or 1, 3, 4, 5 and 6). The downfield 1H peak was due to the proton at positions 2, 4, and 6 (or 2 only). These assignments are consistent with the 1H data reported previously \(^{14}\). It is worth pointing out that there were minor differences between the 1D and 2D 13C resonances. For example, the two resonance peaks of K$_2$H$_{10}$IP$_6$ were 72.2 and 75.3 ppm in the 1D spectrum (Fig. 2), and 74.7 and 75.4 ppm in the 2D spectrum (Fig. 3). We attributed the differences to the approximate nature of deconvolution that did not always set the resonances at the inherent peak values.

The five spectra of non-hydrogen metal phytates show a single contour peak (Fig. 4). The spectra are consistent with mostly 5a/1e conformation in these compounds. For a metal phytate one would potentially have observed more peaks in the HETCOR plot since the 6 ring carbons and 6 protons can show different chemical shift values in solution\(^\text{14, 15, 21}\). However, due to conformational flexibility and limited resolution in solid state NMR, we only observed single HETCOR peaks in Fig. 4 for the five metal phytates. Note that the 2D 1H-13C HETCOR spectral features for hydrogen metal phytates and non-hydrogen metal phytates are different; perhaps the HETCOR spectral pattern can be used to distinguish the phytate compounds from each other. It is noteworthy that the trends we observe in 1D and 2D spectra are similar to those observed for the pH effect. Thus, lower pH (corresponding to 8-10 hydrogens in the hydrogen metal phytates) is associated with 5a/1e conformation, with approximate 4:2 intensity ratio for the 13C peaks at ca. 75 and 72 ppm. Intermediate pH (corresponding to 2-4 hydrogens in the hydrogen metal phytates) is associated with mixed 5a/1e + 1a/5e conformations, with broad, almost merged peaks which can be deconvoluted to give approximately 3:3 intensity ratio for the peaks at ca. 75 and 72 ppm. High pH (corresponding to metal phytates with no hydrogen) corresponds to 1a/5e conformation with a predominant peak at 73 ppm.

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**Figure 4.** 2D 13C-1H heteronuclear correlation (HETCOR) spectra of metal non-hydrogen phytates with 10 ms mixing time.
References


