Nuclear Magnetic Resonance Spectroscopic Analysis of myo-Inositol Phosphates Including Inositol 1,3,4,5-Tetraakisphosphate*

Sebastian Cerdan†, Carl A. Hansen‡, Roy Johanson, Toshio Inubushi, and John R. Williamson§

From the Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6089

(Received for publication, June 30, 1986)

1H and 31P NMR spectra of a variety of phosphorylated myo-inositols have been analyzed using a Bruker WH-360 spectrometer. Proton and phosphorus chemical shifts and coupling constants are reported for myo-inositol 1-phosphate, myo-inositol 2-phosphate, myo-inositol 5-phosphate, myo-inositol 1,2-cyclic phosphate, myo-inositol 1,4-bisphosphate, myo-inositol 1,4,5-trisphosphate, and myo-inositol 1,3,4,5-tetraakisphosphate. These data provide the basis for the chemical identification and characterization of biologically relevant inositol phosphates.

The role of Ins(1,4,5)P3 as an intracellular second messenger involved in agonist-stimulated Ca2+ mobilization has gained widespread support in the last few years and has led to an enormous stimulation of interest in the metabolism of inositol phosphates in mammalian cells (1–3). Recent studies have shown that a number of different inositol polyphosphates are capable of activating a number of mammalian tissues (4–7). Of particular interest is the new finding that Ins(1,4,5)P3 is phosphorylated to Ins(1,3,4,5)P4 by a putative ATP-dependent 3-kinase, which is present in the soluble fraction of a number of mammalian tissues (8, 9). The biological function of Ins(1,3,4,5)P4 is currently unknown, but it has been suggested that this compound is the product of the kinase reaction and of other biologically important inositol phosphates are clearly of importance for a further understanding of their possible role as intracellular second messengers.

13C NMR (10, 11) and 31P NMR (12, 13) techniques have been used to determine the configuration and chemical shifts of inositol isomers and inositol penta- and hexakisphosphates. More recently, high resolution 1H NMR has been used to analyze the structure and conformation of inositol lipids in deuterated methanol (14), whereas Lindon et al. (15) have reported 1H, 13C, and 31P NMR spectra of Ins(1,4,5)P3. The present study was undertaken to provide 1H NMR characterization of several myo-inositol phosphates, including myo-inositol 1,2-cyclic phosphate. The effects of adjacent phosphates on the chemical shifts of the inositol proton have been measured together with the proton-proton and proton-phosphorus coupling constants. 1H and 31P NMR spectra of the product produced by phosphorylation of Ins(1,4,5)P3 by a partially purified inositol-phosphate kinase from rat brain are presented, which allow it to be identified as Ins(1,3,4,5)P4.

EXPERIMENTAL PROCEDURES

Materials—myo-Inositol, myo-inositol 2-phosphate, myo-inositol 1,2-cyclic phosphate, and myo-inositol 1,4-bisphosphate were obtained from Sigma. myo-Inositol 1-phosphate and myo-inositol 5-phosphate were kindly supplied by C. Ballou (University of California, Berkeley) and S. J. Angyal (University of New South Wales, Kensington, Australia), respectively. myo-Inositol 1,4,5-trisphosphate was obtained from Behring Diagnostics, and [2-1H]myo-inositol 1,4,5-trisphosphate was provided by New England Nuclear. Deuterated solvents were purchased from Aldrich and TSP from Merck Sharp and Dohme.

Preparation of Ins(1,3,4,5)P4—Ins(1,4,5)P3 kinase was isolated from rat brain cortex, which was homogenized (1:10, w/v) in buffer containing 25 mM Hepes, pH 8.0, 1 mM MgCl2, 2 mM EGTA, and 1 mM dithiothreitol. The homogenate was centrifuged at 10,000 × g for 10 min, and the supernatant was further centrifuged at 100,000 × g for 60 min to remove membranes. The supernatant was passed through a phosphocellulose column equilibrated with buffer containing 10 mM Tris, pH 8.0, 1 mM MgCl2, and 1 mM dithiothreitol. Bound enzyme was eluted with a linear gradient of 500 mM K2HPO4, pH 8.0, 1 mM dithiothreitol, and the above buffer. After further purification through a hydroxylapatite column, the kinase was essentially free of inositol-phosphate 5-phosphatase activity and had a specific activity of 0.016 mu mol of protein-min at 25 °C. The enzyme was assayed by following the conversion of [3H]Ins(1,4,5)P3 to [3H]Ins(1,3,4,5)P4 using minicolumns of Dowex AG 1- X8 resin and three washes of 0.75 mM NH4COOH, 0.1 mM HCOOH to remove Ins(1,4,5)P3, followed by 2 ml of 1.5 ml of 1.5 mM NH4COOH, 0.1 mM HCOOH to elute Ins(1,3,4,5)P4.

Ins(1,3,4,5)P4 was prepared for NMR spectroscopy by incubating batches of 3 mg of Ins(1,4,5)P3 plus 0.25 μCi of [3H]Ins(1,4,5)P3 in 6 ml of 50 mM Tris, pH 8.0, containing 5 mM ATP, 5 mM Mg2+, 1 mM dithiothreitol with 0.5 mg of protein of Ins(1,4,5)P3 kinase at 37 °C for 1 h. The reaction was stopped by addition of perchloric acid to give a final concentration of 4% (w/v). After neutralization to pH 7.5 and removal of KClO4, the Ins(1,3,4,5)P4 was separated from adenine nucleotides and other inositol phosphates by high performance liquid chromatography using a Pharmacia Mono-Q 5/5 column with a 0–300 mM NH4HCO3 concave gradient. The NH4HCO3 was removed by lyophilization.

NMR Spectroscopy—Samples for NMR spectroscopy (1–3 mg) were dissolved in 0.1 ml of D2O, and pH adjustments were made with NaOD or HCl. High resolution 1H NMR spectra were performed at 360 MHz, pH 8.0, and 25 °C in 5-mm tubes using a Bruker WH-360 spectrometer. The acquisition conditions were as follows: 60° pulses,
NMR Analysis of Inositol Phosphates

3600-Hz spectral width, and 2.27-s acquisition time with accumulation of 128–512 scans. The residual H$_2$O resonance was suppressed by a 2-s selective presaturating pulse. An external standard of TSP contained in a coaxial capillary was used to calibrate $^1$H chemical shifts. Proton-coupled or decoupled $^{31}$P NMR spectra were obtained at 145.8 MHz using 45° pulses at 2-s intervals, 10,000-Hz spectral width, and an acquisition time of 0.85 s. Phosphorus chemical shifts are reported relative to external methylenediphosphonic acid at 18.623 ppm from the 85% orthophosphoric acid resonance defined as 0 ppm. For determination of coupling constants, free induction decays were zero-filled to 32,000 and multiplied by a gaussian window for resolution enhancement, which gave a digital resolution of 0.1-0.2 Hz. Coupling constants and proton chemical shift measurements were derived initially from the first-order analysis of the $^1$H NMR spectra and further optimized by iterative procedures using the Parameter Adjustment in NMR by Iteration Calculation (PANIC) program. The coupling constants associated with these interactions as determined from the proton-coupled $^{31}$P NMR spectra were as follows, $J_{	ext{H(1)-P}} = 7.3$ Hz and $J_{	ext{H(2)-P}} = 8.5$ Hz. These heteronuclear coupling constants are of similar magnitude to the homonuclear coupling constants between equatorial protons (Table II) and produce a triplet of doublets structure for the H-1 of Ins-1-P and a doublet of triplets structure for the H-2 of Ins-2-P.

**RESULTS AND DISCUSSION**

In order to provide a basis for interpretation of the $^1$H NMR spectra of the higher inositol phosphates, the effects of monophosphate substitution in the myo-inositol ring on proton chemical shifts and coupling constants were evaluated using Ins-1-P, Ins-2-P, Ins-5-P, and Ins(1,2)cyclic-P. The proton chemical shifts for these compounds are given in Table I, whereas proton-proton coupling constants are shown in Table II.

The splitting patterns of the individual proton resonances due to vicinal coupling of each proton with neighboring nuclei are illustrated in Fig. 1A for myo-inositol and in Fig. 1B for Ins-1-P. Because of the symmetry of the myo-inositol ring about the H(2)-H(5) axis, only four discrete proton resonances are observed in free myo-inositol. The H-2, H-4(H-6) and H-5 resonances are split into triplet-like structures due to the fact that the vicinal H-H coupling constants are of similar magnitude; those for the H-1(H-3) resonances are dissimilar, and consequently these proton resonances have a doublet of triplets structure. Differences in the H-H coupling constants (see Table II) reflect the fact that the hydroxyl at position 2 of the myo-inositol ring is axial, whereas the remaining hydroxyl groups are equatorial (16). Phosphorylation at position 1 destroys the symmetry of the inositol ring and causes an increase of the chemical shift of the H-1 proton by about 0.4 ppm (Fig. 1B). Small increases (0.15–0.18 ppm) of the chemical shifts of the adjacent protons (H-2 and H-6) were also produced, and all six proton resonances appear as discrete multiplets. The sample of Ins-1-P used in the present study contained a small amount of Ins-2-P, which could readily be identified from the $^1$H NMR spectrum. The small resonance at about 4.5 ppm (Fig. 1B), which had a chemical shift 0.44 ppm greater than that of the H-2 of inositol, can be ascribed to the H-2 proton of Ins-2-P (cf. Table I). The H-2 of authentic Ins-2-P is shown in the inset and has a characteristic doublet of triplets structure. Additional resonances due to Ins-2-P are seen centered at 3.25 ppm (H-5), 3.46 ppm (H-1, H-3), and 3.72 ppm (H-4, H-6). Further splitting of the H-1 and H-2 resonances in Ins-1-P and Ins-2-P, respectively, is due to coupling of these protons with $^{31}$P. The coupling constants associated with these interactions as determined from the proton-coupled $^{31}$P NMR spectra were as follows, $J_{	ext{H(1)-P}} = 7.3$ Hz and $J_{	ext{H(2)-P}} = 8.5$ Hz. These heteronuclear coupling constants are of similar magnitude to the homonuclear coupling constants between equatorial protons (Table II) and produce a triplet of doublets structure for the H-1 of Ins-1-P and a doublet of triplets structure for the H-2 of Ins-2-P.

**myo-Inositol 1,2-cyclic phosphate produces a very different $^1$H NMR spectra, as illustrated in Fig. 2B. The H-1 and H-2 protons are both shifted by 0.79 ppm relative to myo-inositol (see Table I), although the H-2 resonance shown in Fig. 2B was slightly distorted by the irradiation used to suppress the nearby H$_2$O signal. The H-1 proton resonance is split into a multiplet (doublet of quartets) due to heteronuclear coupling to the phosphorus nucleus with an unusually large coupling constant of 19.5 Hz, indicative of a greater degree of covalent bonding, and to different homonuclear coupling constants ($J_{	ext{H(1)-H(2)}} = 4.1$ Hz, $J_{	ext{H(1)-H(3)}} = 8.3$ Hz). Unexpectedly, the H-3 proton resonance exhibited a similar multiplet structure. Selective homonuclear proton decoupling experiments at the H-2 or H-4 resonance reduced the multiplicity of the H-3 resonance to a doublet of doublets structure, indicating a long range interaction between the H-3 proton and the phosphorus nucleus with an estimated coupling constant of 1.9 Hz. This phenomenon, together with the increase in the magnitude of $J_{	ext{H(1)-H(2)}}$ and $J_{	ext{H(1)-H(3)}}$ from $J_{	ext{H(1)-H(2)}}$, 3.0 to 4.1 Hz upon formation of the five-membered cyclic phosphodiester ring probably reflects a significant distortion of the normal myo-inositol chair conformation. In addition, the proton-decoupled $^{31}$P NMR spectrum of Ins(1,2)cyclic-P showed a singlet resonance far downfield at 16.77 ppm. By analogy with other five-membered cyclic phosphates, which also exhibit a large downfield shift, an O-P-O bond angle of 98–100° is estimated (17); this is considerably smaller than the tetrahedral angle of $109^\circ$. Upon alkaline hydrolysis of Ins(1,2)cyclic-P, two $^{31}$P resonances appeared in the approximate proportions of 2:1 in accordance with previous findings (18), corresponding to Ins-1-P at 4.75 ppm and Ins-2-P at 5.26 ppm. Confirmatory evidence for the proton chemical shift assignments and the multiplet structures was obtained by simulation of the $^1$H NMR spectrum of Ins(1,2)cyclic-P as depicted in Fig. 2A. The $^1$H NMR spectrum of Ins(1,4)P$_2$ is shown in Fig. 3A; the addition of phosphate at position 4 to Ins-1-P increased

<table>
<thead>
<tr>
<th>myo-InsP</th>
<th>Insotol</th>
<th>Ins-1-P</th>
<th>Ins-2-P</th>
<th>Ins-5-P</th>
<th>Ins(1,2)cyclic-P</th>
<th>Ins(1,4)P$_2$</th>
<th>Ins(1,4,5)P$_3$</th>
<th>Ins(1,3,4,5)P$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-1</td>
<td>3.52$^d$</td>
<td>3.90$^d$</td>
<td>3.46$^d$</td>
<td>3.60$^d$</td>
<td>4.32$^q$</td>
<td>$\sim$3.90$^a$</td>
<td>4.00$^e$</td>
<td></td>
</tr>
<tr>
<td>H-2</td>
<td>4.05$^a$</td>
<td>4.23$^a$</td>
<td>4.49$^a$</td>
<td>4.04$^d$</td>
<td>4.84$^q$</td>
<td>4.23$^a$</td>
<td>4.30$^a$</td>
<td>4.42$^a$</td>
</tr>
<tr>
<td>H-3</td>
<td>3.58$^q$</td>
<td>3.58$^q$</td>
<td>3.46$^q$</td>
<td>3.60$^q$</td>
<td>3.75$^q$</td>
<td>3.67$^q$</td>
<td>3.73$^q$</td>
<td>4.08$^q$</td>
</tr>
<tr>
<td>H-4</td>
<td>3.59$^q$</td>
<td>3.64$^q$</td>
<td>3.72$^q$</td>
<td>$\sim$3.75$^a$</td>
<td>3.64$^q$</td>
<td>4.13$^q$</td>
<td>4.21$^q$</td>
<td>4.38$^q$</td>
</tr>
<tr>
<td>H-5</td>
<td>3.57$^q$</td>
<td>3.34$^q$</td>
<td>3.25$^q$</td>
<td>$\sim$3.75$^a$</td>
<td>3.29$^q$</td>
<td>3.48$^q$</td>
<td>$\sim$3.94$^q$</td>
<td>4.01$^q$</td>
</tr>
<tr>
<td>H-6</td>
<td>3.59$^q$</td>
<td>3.74$^q$</td>
<td>3.72$^q$</td>
<td>$\sim$3.75$^a$</td>
<td>3.82$^q$</td>
<td>3.83$^q$</td>
<td>$\sim$3.96$^q$</td>
<td>3.90$^q$</td>
</tr>
</tbody>
</table>

The spectra were recorded as described under "Experimental Procedures." Chemical shifts are expressed in parts/million relative to TSP contained in a coaxial capillary at 0 ppm. The superscripts refer to the observed multiplicity of the proton resonances: dd, doublet of doublets; t, triplet; td, triplet of doublets; dt, doublet of triplets; u, unresolved; dq, doublet of quartets; q, quartet. The symbol $\sim$ refers to approximate chemical shift.
NMR Analysis of Inositol Phosphates

Table II

Proton-proton vicinal coupling constants of myo-inositol phosphates

<table>
<thead>
<tr>
<th>$J_{HH}$</th>
<th>Insolit</th>
<th>Ins-1-P</th>
<th>Ins-2-P</th>
<th>Ins-5-P</th>
<th>Ins(1,2)cyclic-P</th>
<th>Ins(1,4)P$_3$</th>
<th>Ins(1,4,5)P$_3$</th>
<th>Ins(1,3,4,5)P$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(1)-H(2)</td>
<td>3.0</td>
<td>2.3</td>
<td>2.4</td>
<td>2.9</td>
<td>4.1</td>
<td>2.9</td>
<td>2.8</td>
<td>2.6</td>
</tr>
<tr>
<td>H(2)-H(3)</td>
<td>3.0</td>
<td>2.3</td>
<td>2.4</td>
<td>2.9</td>
<td>4.1</td>
<td>2.9</td>
<td>2.8</td>
<td>2.6</td>
</tr>
<tr>
<td>H(3)-H(4)</td>
<td>10.1</td>
<td>9.8</td>
<td>10.1</td>
<td>9.4</td>
<td>10.0</td>
<td>9.7</td>
<td>9.6</td>
<td>9.8</td>
</tr>
<tr>
<td>H(4)-H(5)</td>
<td>9.4</td>
<td>9.8</td>
<td>9.6</td>
<td>9.4</td>
<td>10.1</td>
<td>9.4</td>
<td>9.6</td>
<td>9.0</td>
</tr>
<tr>
<td>H(5)-H(6)</td>
<td>9.4</td>
<td>7.0</td>
<td>9.8</td>
<td>9.3</td>
<td>10.0</td>
<td>9.5</td>
<td>9.6</td>
<td>9.4</td>
</tr>
<tr>
<td>H(6)-H(1)</td>
<td>10.1</td>
<td>6.6</td>
<td>10.1</td>
<td>9.4</td>
<td>8.3</td>
<td>9.3</td>
<td>9.5</td>
<td>9.8</td>
</tr>
</tbody>
</table>

The $^1$H NMR spectra (360 MHz) of myo-inositol (A) and myo-inositol 1-phosphate (B). The splitting pattern of the different proton resonances due to spin-spin interactions between neighboring protons or phosphorus nucleus is illustrated above each proton multiplet resonance. The inset in B (H-2') depicts the H-2 resonance of myo-inositol 2-phosphate. The spectrum were obtained using an 8000 data table with 311 scans for A and 1215 scans for B.

**Fig. 1.** $^1$H NMR spectra (360 MHz) of myo-inositol (A) and myo-inositol 1-phosphate (B). The resonances in Fig. 1B caused the H-5 proton also to shift downfield by about 0.5 ppm, thereby accounting for the prominent peak at 3.93 ppm in the $^1$H NMR spectra of Ins(1,4,5)P$_3$, which is a composite of the H-5, H-6, and H-1 resonances. The $^1$H NMR spectrum shown in Fig. 3B contains additional resonances attributable to Ins(2,4,5)P$_3$. These were identified by (a) the doublet of triplets resonance at 4.48 ppm due to the H-2 proton coupled to phosphorus, (b) the multiplet centered at 4.12 ppm, which corresponds to the H-4 proton coupled to phosphorus, and (c) the doublets resonance centered at 3.52 ppm due to the H-1 proton.

**Fig. 2.** $^1$H NMR spectra (360 MHz) of myo-inositol 1,2-cyclic phosphate. A, simulated spectrum; B, experimental spectrum. The resonances “X” in B is due to methanol. A 16,000 data table was used for 128 scans.

The multiplicity of the H-4 proton from a triplet-like structure to that of a complex quartet and caused the H-4 resonance to shift downfield by about 0.5 ppm (cf. Fig. 1B). Selective proton decoupling of the proton-coupled $^3$P NMR spectra of Ins(1,4)P$_3$ allowed the assignment of P-4 and P-1 at 5.35 and 4.90 ppm, respectively, with $J_{H-4-P} = 7.3$ Hz and $J_{H-1-P} = 7.6$ Hz. Further phosphorylation of Ins(1,4)P$_3$ at position 5 (Fig. 3B) caused the H-5 proton to shift downfield by about 0.5 ppm, thereby accounting for the prominent peak at 3.93 ppm in the $^1$H NMR spectrum of Ins(1,4,5)P$_3$, which is a composite of the H-5, H-6, and H-1 resonances. The $^1$H NMR spectrum shown in Fig. 3B contains additional resonances attributable to Ins(2,4,5)P$_3$. These were identified by (a) the doublet of triplets resonance at 4.48 ppm due to the H-2 proton coupled to phosphorus (see inset for the H-2 of Ins-2-P in Fig. 1B), (b) the multiplet centered at 4.12 ppm, which corresponds to the H-4 proton coupled to phosphorus, and (c) the doublets resonance centered at 3.52 ppm due to the H-1 proton.

The $^1$H NMR spectrum at pH 8.0 of the product isolated from the action of brain myo-inositol-trisphosphate kinase on Ins(1,4,5)P$_3$ is shown in Fig. 4A. The assignments of the resonances in Fig. 4A were determined by homonuclear and heteronuclear decoupling experiments, e.g. saturation of the triplet of doublets resonance ascribed to H-3 collapsed the H-4 quartet centered at 4.38 ppm to a triplet and collapsed the H-2 triplet centered at 4.42 ppm to a doublet with a coupling constant of 2.6 Hz. Likewise, saturation of the H-4 resonance converted the H-3 resonance to a doublet of doublets and converted the mixed H-1 and H-5 resonances to a well-resolved doublet of triplets (H-1) and a triplet centered at 4.01 ppm (H-5), whereas saturation of the H-2 resonance...
converted the triplet of doublets resonance of H-3 to a triplet centered at 4.08 ppm. The proton chemical shifts for Ins(1,3,4,5)P4 and the proton-proton coupling constants are given in Tables I and II, respectively.

The proton-coupled 31P spectrum of Ins(1,3,4,5)P4, at pH 8.0, is shown in Fig. 4B. Four doublet resonances are seen centered at 3.77, 3.18, 2.75, and 2.50 ppm. Selective heteronuclear proton decoupling at the frequencies associated with the H-4 and H-3 resonances (cf. Fig. 4A) indicated that the resonances at 2.50 and 2.75 ppm originated from phosphorus nuclei located at positions 4 and 3, respectively, of the inositol ring. Further experiments with selective 31P decoupling of the 31P NMR spectra confirmed that decoupling of P-4 converted the H-4 resonance from a quartet to a triplet, whereas decoupling of P-3 converted the H-3 resonance from a quartet to a triplet, whereas decoupling of P-3 and P-4 converted the H-3 resonance from a quartet to a triplet, whereas decoupling of P-3 and P-4 converted the H-3 resonance from a quartet to a triplet, whereas decoupling of P-3 and P-5 permitted the assignments for these phosphorus resonances to be made, as shown in Fig. 4B. The proton-coupled 31P NMR spectrum allowed values of 7.7, 9.4, 9.3, and 8.7 Hz to be calculated for $J_{H_1-P}$, $J_{H_3-P}$, $J_{H_4-P}$, and $J_{H_5-P}$, respectively. These proton-phosphorus coupling constants are very similar to those of Ins-1-P, Ins(1,4)P2, and Ins(1,4,5)P3 and to the proton-proton coupling constants, exclusive of the H-2 proton, given in Table II for the different inositol phosphates. The low value of 3.4 Hz for the proton-phosphorus coupling constant of P-1 or P-5 of Ins(1,4,5)P3, reported by Lindon et al. (15) was not confirmed in our study. An unusual feature of the 31P NMR spectra of the inositol phosphates is that the addition of the extra phosphate in position 3 to Ins(1,4,5)P3 causes a large upfield shift of the P-4 resonance.

From a knowledge of the chemical shifts of individual protons of inositol when mono- and polyphosphorylated at different positions (Table I), a tentative algorithm describing the effects of phosphorylation on the chemical shifts of the different protons in the inositol ring can be derived as follows.

$$\delta H-x(IP) = \delta H-x(I) + n_x \alpha + n_y \beta + n_z \gamma$$

$\delta H-x(IP)$ is the chemical shift of a particular proton in a given inositol phosphate, $\delta H-x(I)$ is the chemical shift of the same proton in myo-inositol, and $\alpha$, $\beta$, and $\gamma$ are empirical constants obtained from the 1H chemical shift data in Table I for inositol phosphates phosphorylated at equatorial positions. These constants define the effect of phosphorylation on the chemical shift of protons in the same position (x), adjacent positions (y), and two positions removed (z) from the phosphorylated site. Mean ± S.E. values are: $\alpha = 0.44 ± 0.02$, $\beta = 0.18 ± 0.01$, and $\gamma = 0.02$. Numerical values for $n_x$ are 0 or 1, whereas $n_y$ and $n_z$ can be 1 or 2 (independently) depending on the position and number of phosphates in the inositol phosphate. When the axial hydroxyl is phosphorylated (position 2), the chemical shift of adjacent protons is decreased (upfield rather than downfield shift), and the above formula does not apply.

REFERENCES
NMR Analysis of Inositol Phosphates