¹H, ¹³C and ³¹P n.m.r. spectra and molecular conformation of *myo*-inositol 1,4,5-trisphosphate

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Multinuclear n.m.r. spectroscopy has been used to confirm the identity of inositol 1,4,5-trisphosphate and to deduce its molecular conformation as having five equatorial and one axial ring substituents.

INTRODUCTION

In an elegant and comprehensive series of experiments over 20 years ago, Ballou and co-workers (Grado & Ballou, 1961; Tomlinson & Ballou, 1961) completely characterized the *myo*-inositol polyphosphates from brain phospholipids, in particular Ins(1,4,5)₃. This substance has recently achieved a great deal of attention as an important cellular second messenger (Berridge & Irvine, 1984), but despite this no spectroscopic evidence has been presented for its identity. The present paper describes recent n.m.r. results which are consistent with Ballou's structure, lists the appropriate spectroscopic parameters and shows the ring conformation to exist in a form in which five substituents are equatorial and only one axial over the pH range 6–9.5:

EXPERIMENTAL

The sample of Ins(1,4,5)₃ was a gift from Dr. R. F. Irvine of the Agricultural and Food Research Council, Institute of Animal Physiology, Babraham, Cambridge, U.K. (Irvine *et al.*, 1984). The material (approx. 400 µg) was dissolved in ²H₂O to a concentration of approx. 2 mM in a 5 mm n.m.r. tube and used without further treatment other than solution 'pH' adjustment using NaO²H or ²HCl. N.m.r. spectra were measured using a Bruker AM-360 spectrometer at 360.13 MHz for ¹H, 145.78 MHz for ³¹P and 90.57 MHz for ¹³C. Details of each spectrum are quoted in the Figure captions. All spectra were obtained at ambient probe temperatures of approx. 21 °C for ¹H and ³¹P and approx. 40 °C for ¹H broadband decoupled ¹³C n.m.r.

RESULTS

The ¹³C spectrum acquired overnight at pH approx. 6 gave only a low signal-to-noise ratio but served to identify six non-equivalent resonances all in the chemical shift

region expected for –CHO– carbons (Fig. 1a). The shifts are listed in Table 1. Assignments have been made on the basis of the α- and β-substituent effect of phosphorylation on the ¹³C n.m.r. shifts of *myo*-inositol (Johnson & Jankowski, 1972), using values given for phosphodiester formation as an approximation (Poxton *et al.*, 1978). Having assigned the peaks as in Table 1, a regression analysis yielded adjusted values for the α-phosphorylation effect as +3.4 p.p.m. and for the β effect as –0.5 p.p.m. These parameters then allowed the prediction of all of the observed shifts to within 0.4 p.p.m. The resolution and signal-to-noise ratio precluded observation of the expected ¹³C–³¹P spin-coupling constants.

The ³¹P n.m.r. spectrum was poorly resolved at pH approx. 6, probably due to the phosphate groups being in an intermediate state of protonation, and showed two major resonances one of which was apparently concealing a broad band attributable to a titrating phosphate. However at pH approx. 9.5 all three phosphate signals were clearly resolved and the ³¹P–¹H three bond couplings could be measured. Broadband ¹H decoupling caused all signals to collapse to singlets. The spectrum is shown in Fig. 1(b) and the relevant parameters are given in Table 1.

The ¹H spectrum, also at pH approx. 9.5, is shown in Fig. 1(c) and is again in agreement with the structure of Ins(1,4,5)₃. Resonances corresponding to the six –CH– groups are observed, the highest frequency signal at δ4.28 being assignable to H-2, coupling to H-3 with *J*_{H–H} 2.6 Hz typical for an axial–equatorial interaction. The resonance for H-3 appears at δ3.70 as a doublet of doublets with, in addition to the H-2–H-3 interaction, a second coupling to H-4 of 9.6 Hz, typical of an axial–axial environment. In turn the resonance for H-4 is found at δ4.18 with a quartet-like structure indicating three approximately equal couplings of approx. 10 Hz, i.e. to H-3, H-5 and the attached phosphate ³¹P. All of these H–H connectivities have been confirmed using spin decoupling; for example, irradiation at the remaining band at δ approx. 3.91 arising from three proton signals causes the removal of one coupling from the resonance at δ4.18 and confirms the H-4–H-5 coupling also to be typical of an axial–axial interaction. Additional confirmation of the assignments made so far comes from a two-dimensional ³¹P–¹H chemical shift correlation experiment with connectivities being deduced

Fig. 1. N.m.r. spectra of Ins(1,4,5) P_3

(a) ^{13}C spectrum: 26320 scans, spectrum width 20 kHz, 32768 data points, Fourier transform size 32768, line broadening 10 Hz, broadband ^1H decoupling, pH approx. 6. (b) ^{31}P spectrum: 57646 scans, spectrum width 12.5 kHz, Lorentzian–Gaussian resolution enhancement, 16384 data points, Fourier transform size 65536, pH approx. 9.5. (c) ^1H spectrum: 128 scans, spectrum width 5 kHz, 32768 data points, Fourier transform size 32768, Lorentzian–Gaussian resolution enhancement, pH approx. 9.5.

using the three bond ^{31}P – ^1H coupling constant (Bodenhausen & Freeman, 1977). The results are presented as a contour map and are shown in Fig. 2. Clearly the ^1H chemical shift of H-4 at δ 4.18 is correlated with the ^{31}P

Table 1. N.m.r. parameters for Ins(1,4,5) P_3

Chemical shifts are labelled according to the nucleus being observed and the position number in the *myo*-inositol ring. ^{13}C shifts are in p.p.m. quoted relative to tetramethylsilane but referenced to external dioxan at 67.4 p.p.m. ^{31}P shifts are in p.p.m. quoted relative to external H_3PO_4 . ^1H shifts (δ) are quoted relative to tetramethylsilane but referenced to ^2HHO at 4.8 at pH 9.5. Coupling constants are in Hz, by first order analysis.

Chemical shifts		Coupling constants (Hz)	
C-1	76.3	$J_{\text{H-2-H-3}}$	2.6
C-2	72.2	$J_{\text{H-3-H-4}}$	9.6
C-3	73.0	$J_{\text{H-4-H-5}}$	approx. 7.6
C-4	75.0		
C-5	78.4	$J_{\text{H-1-P}}$	3.4†
C-6	71.6	$J_{\text{H-4-P}}$	7.6
P-1	5.59*	$J_{\text{H-5-P}}$	6.9†
P-4	7.25		
P-5	7.10*		
H-1	approx. 3.91		
H-2	4.28		
H-3	3.70		
H-4	4.18		
H-5	approx. 3.91		
H-6	approx. 3.91		

*† Assignment pairs may be reversed.

shift at 7.25 p.p.m. and serves to assign this shift to P-4. Similarly the other two phosphorus shifts correlate with ^1H chemical shifts in the three-proton multiplet at δ approx. 3.91 and it is not possible to assign the phosphorus resonances unambiguously, although P-1 is not adjacent to another phosphate like the P-4/P-5 pair and may thus be giving rise to the shifted resonance at 5.59 p.p.m. As expected neither the H-2 nor H-3 signals correlate in the phosphorus spectrum. This rules out any possibility of phosphate substitution at C-2 or C-3.

The ^1H chemical shifts and coupling constants measured here agree quite closely with those found (Shibata *et al.*, 1984) for the equivalent phosphoinositide phospholipid in methanol, except that the observed shifts for H-4 and H-2 are reversed. Also in that case H-1, H-5 and H-6 resonances could not be resolved. In both cases H-2 could be resolved but no measurement of $J_{\text{H-1-H-2}}$ was possible. In the present study the maximum value of any splitting due to this coupling is within the linewidth (approx. 1.5 Hz) and thus either the coupling is smaller than this or second order effects due to the small chemical shifts in the H-1, H-5, H-6 band are operating.

The resolved ^1H coupling constants are unchanged over the pH range 6–9.5, showing that under these circumstances there is no conformational ring flip and that all phosphates are equatorial. This conformation is also found in the intact phospholipid in methanol (Shibata *et al.*, 1984). This contrasts with *myo*-inositol hexakisphosphate which undergoes a pH-dependent conformational change from five equatorial to five axial phosphates at pH approx. 12, which conformation is maintained to about pH 5 when it reverts again to the equatorial form (Emsley & Niazi, 1981).

In addition to the axial/equatorial nature of the ring

Fig. 2. Two-dimensional ^1H - ^{31}P chemical shift correlation contour map showing connectivities via $^3J_{\text{H-P}}$

256 scans for each of 256 experiments with 1024 data points, spectrum width 1259 Hz; zero-filled to 1024 in F1 domain (^1H axis) with Lorentzian-Gaussian enhancement in both.

substituents, some information can be gleaned on the populated rotameric forms of the phosphate groups, based on the values of the three bond ^{31}P - ^1H spin couplings, which lead to estimates of the H-C-O-P dihedral angle. Recently it has been shown that the dihedral angle dependence of this coupling constant follows a Karplus-type function (Lankhorst *et al.*, 1984) with J_{0° approx. 11 Hz, J_{60° approx. 2.5 Hz and J_{180° approx. 23 Hz. Consequently, for the 1-phosphate in

Ins(1,4,5) P_3 which exhibits a coupling constant of 3.4 Hz any significant contribution from the 180° form, i.e. phosphate *trans* to the C-1-H-1 bond, can be ruled out and the most likely conformation is one with the phosphate O-P bond at an angle of about 50 - 60° (gauche in the terminology of Emsley & Niazi, 1981) to the C-H bond, a favourable position for hydrogen-bonding to the OH at C-6. There is no ability of the phosphate at C-1 to hydrogen bond to the OH at C-2 which would necessitate an antiperiplanar (i.e. 180°) arrangement to the C-1-H-1 bond. Similarly, the couplings H-4-P-4 and H-5-P-5, which are 7.6 Hz and 6.9 Hz, could be explained as being due to a slightly weighted rotational average of all three possible staggered rotamers about the C-O bond which for free rotation would amount to a coupling of 9.3 Hz. Alternatively, they could reflect the fact that the predominant rotamer at pH 9.5 has a dihedral angle of 30 - 35° or 115 - 120° . Without recourse to other data such as ^{13}C - ^{31}P coupling constants it is not possible to be more definite.

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