Isotope-induced Nonequivalence in a Symmetrical Molecule: Measurement of the ^{31}P–^{31}P Geminal Coupling Constant in Pyrophosphate

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Asymmetric incorporation of ^{18}O into pyrophosphate to perturb the ^{31}P n.m.r. resonance frequency allows determination of the ^{31}P–^{31}P geminal coupling constant in this otherwise symmetrical molecule.

We report the use of selective ^{18}O labelling of one phosphorus atom in pyrophosphate to induce chemical shift non-equivalence of the two phosphorus nuclei and thus permit observation of the individual resonances and measurement of an otherwise unobservable mutual coupling constant.

^{18}O induces a small upfield shift in the ^{31}P n.m.r. resonance frequency of phosphates (*ca.* 0.02 p.p.m./^{18}O bond), the magnitude of which is related to the P–O bond order.[1–3] Recent work in this laboratory involving the synthesis of ^{18}O-β,γ-bridge-labelled ATP for use in positional isotope exchange experiments produced an interesting result while assigning resonances in the ^{31}P n.m.r. spectra of ^{18}O-labelled pyrophosphate intermediates.[4] At 202.5 MHz, the ^{31}P n.m.r. spectrum of pyrophosphate with one nonbridging ^{18}O appeared as a closely spaced doublet with a separation of 0.5 Hz. This splitting was presumed to be due to coupling between the nonequivalent phosphorus atoms with the observed doublet corresponding to the central doublet of an AB pattern. By selectively increasing the number of ^{18}O atoms attached to one of the two phosphorus atoms in the pyrophosphate molecule we can increase the nonequivalence of the phosphorus atoms sufficiently to allow observation of

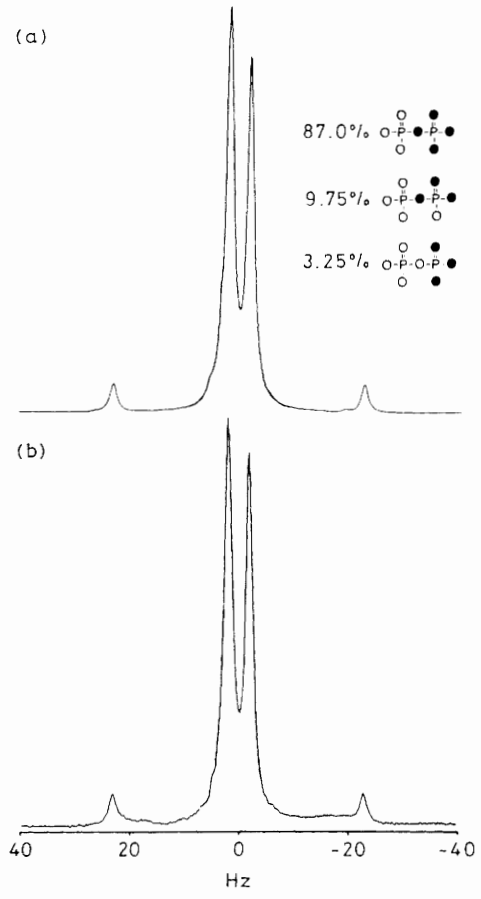

Figure 1. (a) Simulated ^{31}P n.m.r. spectrum of $^{18}O_4$- and $^{18}O_3$-labelled pyrophosphate generated by summing weighted subspectra of the illustrated compounds. Simulations were done on a Nicolet 1180 computer using the Nicolet program NTCSIM. Linewidths were set to 1.5 Hz and a coupling constant of 21.1 Hz was used in all simulations. Chemical shifts were calculated as described in the text. (b) ^{31}P N.m.r. spectrum at 202.5 MHz of [α-$^{18}O_4$]pyrophosphate contaminated with 13% [α-$^{18}O_3$]pyrophosphate. The n.m.r. sample was prepared by dissolving the tetrasodium salt (19 mg) in 0.4 ml of 50% D_2O containing 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid. A 5 mm acid-washed n.m.r. tube was used. The spectrum was obtained using a Bruker 500 MHz spectrometer located at the Southern California Regional NMR Facility, California Institute of Technology. The sample was spun at 17 $\pm$ 1 Hz and 128 accumulations were made using a spectral width of 2000 Hz. The free induction decay was collected using 8192 data points followed by zero-filling to 16 K before Fourier transformation.

the outer resonances of the AB pattern. From this pattern the ^{31}P–^{31}P geminal coupling constant in pyrophosphate can be easily determined.

[α-$^{18}O_4$]Pyrophosphate was prepared from [γ-$^{18}O_4$]ATP using yeast acetyl coenzyme A synthetase.[5,6] [γ-$^{18}O_4$]ATP was prepared by the method of Hoard and Ott[7] from ADP and [$^{18}O_4$]phosphate [prepared, in turn, from $H_2{}^{18}O$ (99%) and PCl_5].[8] The isotopic purity of the [$^{18}O_4$]phosphate was determined by mass spectral analysis of the trimethyl ester derivative, which was prepared by methylation of the free acid with diazomethane in diethyl ether.[9–11]

Figure 1(b) shows the 202.5 MHz spectrum of α-$^{18}O_4$-labelled pyrophosphate. The ^{31}P–^{31}P geminal coupling constant in this AB pattern is simply the separation between either the two downfield or the two upfield peaks and was measured to be 21.1 Hz, a value comparable to the ^{31}P–^{31}P coupling constants found in ATP.[12] The uncoupled chemical shift difference of the two phosphorus resonances was calculated to be 13.3 Hz (at 202.5 MHz) for our labelled pyrophosphate.[13] Based on a net difference of four P–^{18}O bonds, the observed chemical shift difference gives an upfield shift due to ^{18}O of 0.0164 p.p.m./bond, which is consistent with published values.[1] The unequal peak heights of the central doublet and the shoulder appearing on the downfield side of the central doublet were attributed to the presence of contaminating [α-$^{18}O_3$]pyrophosphate. Figure 1(a) shows the simulated spectrum generated from three separately simulated subspectra which were weighted appropriately and then summed. Chemical shifts of the pyrophosphates were calculated based on the experimentally determined upfield shift of 0.0164 p.p.m./^{18}O bond. In the case of ^{18}O-bridged [α-$^{18}O_3$]pyrophosphate the chemical shift difference used in the simulation was calculated using 3.67 ^{18}O bonds to the α-phosphorus (an average of the three resonance forms).

Since scalar coupling constants between equivalent nuclei cannot be determined using magnetic resonance experiments,[14,15] the magnetic environment of one or more of the nuclei must be altered, usually by chemically modifying the molecule. The use of isotope-induced chemical shift perturbations as illustrated in this work permits the measurement of coupling constants between equivalent nuclei without modification of either structure or chemistry. Oxygen isotope-effects (specifically, ^{16}O and ^{18}O) are especially useful in this respect since the observed isotope-effect is due solely to a change in mass. Complications arising from changes in the spin quantum number (giving rise to additional coupling) can therefore be avoided.

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