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Proton Exchange in Type II Isopentenyl Diphosphate Isomerase

Sam J. Barkley, Shrivallabh B. Desai, and C. Dale Poulter*

Department of Chemistry, University of Utah, 315 South 1400 East, RM 2020, Salt Lake City, Utah 84112-0850

poulter@chemistry.utah.edu

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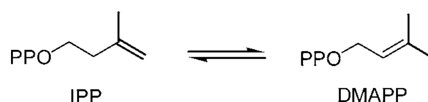
ABSTRACT



Type II isopentenyl diphosphate:dimethylallyl diphosphate (IPP:DMAPP) isomerase from *Synechocystis* PCC 6803 catalyzes the interconversion of IPP and DMAPP. Upon incubation of the enzyme with IPP or DMAPP in $^{2}H_2O$, one deuterium is incorporated into the C2 methylene of IPP, two deuteriums are incorporated at C4, and three deuteriums are incorporated into the (*E*)-methyl of DMAPP.

Isopentenyl diphosphate:dimethylallyl diphosphate (IPP: DMAPP) isomerase catalyzes the interconversion of the two fundamental five-carbon building blocks of the isoprenoid biosynthetic pathway. Two protein families have been identified with IPP isomerase activity. The type I enzyme was discovered in the 1950s and has been studied extensively since that time.[1,2] The type II enzyme was first reported in 2001.[3] Proteins from the two families are structurally unrelated and appear to operate by different chemical mechanisms. Type I IPP isomerase catalyzes an antarafacial transposition of hydrogen between C2 and C4 by protonation of the double bonds in IPP and DMAPP,[4] followed by elimination of a proton from the tertiary cationic intermediate (see Scheme 1).[5,6] The only cofactor required by the enzyme is a divalent metal ion. The mechanism for the type II isomerase has not been determined. However, the enzyme requires a reduced flavin cofactor in addition to a divalent metal for activity. The stereochemistry of the reaction catalyzed by type II IPP isomerase from *Bacillus subtilis* appears to be similar to that of the type I enzyme.[7] We now report NMR and MS experiments that suggest that the stereoselectivity of type II IPP isomerase from *Synechocystis* PCC 6803[8] is higher than that of the type I protein.

Scheme 1

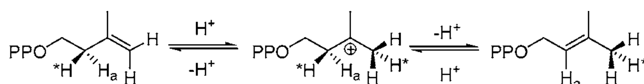


Figure 1 shows the ^{1}H NMR spectrum of an equilibrium mixture of IPP and DMAPP (part A) and expansions of diagnostic peaks for IPP (part C) and DMAPP (parts B and D) after incubation in H_2O. The conversion of IPP to DMAPP gave signals for DMAPP at 5.48 (a one-proton triplet for the hydrogen at C2), 1.77 (a three-proton singlet for the (*E*)-methyl group at C3), and 1.72 ppm (a three-proton singlet for the (*Z*)-methyl group at C3) and the concomitant decrease in the intensities of the signals for IPP at 2.40 (a two-proton triplet for the methylene protons at C2) and 1.775 ppm (a three-proton singlet for the methyl group at C3).[9] The (*E*)-methyl in DMAPP and the methyl in IPP are not sufficiently well-resolved to measure the relative concentrations of IPP and DMAPP in the equilibrium mixture. The protons at C1 in IPP and DMAPP were obscured by signals

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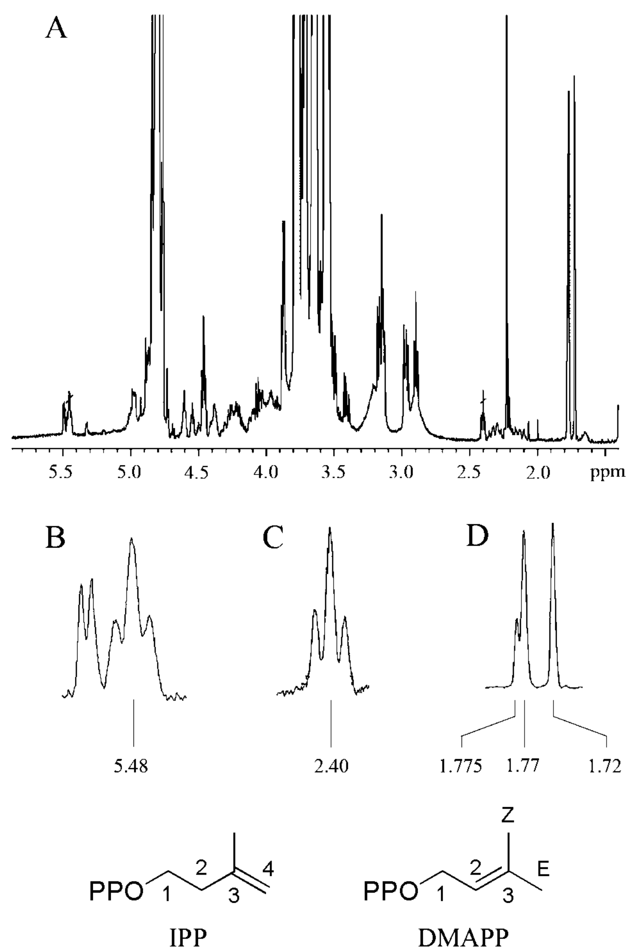


Figure 1. ^{1}H NMR spectra of the reaction mixture after equilibration of IPP with type II IPP isomerase in H_2O. Samples contained 2 mM IPP or DMAPP, 20 μM flavin mononucleotide (FMN), 5 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 20 mM $MgCl_2$, and 4 μM *Synechocystis* PCC 6803 type II IPP isomerase in 100 mM phosphate buffer, pH 7.0, in a total volume of 1.0 mL and were incubated at 37 °C for 16 h. The samples were lyophilized and resuspended in ^2H_2O. Part A: ^{1}H spectrum of the mixture. Parts B−D are expansions of selected resonances for IPP and DMAPP. Part B: C2 proton in DMAPP (5.48 ppm). Part C: C2 protons in IPP (2.40 ppm). Part D: methyl group in IPP (1.775 ppm), (*E*)-methyl in DMAPP (1.77 ppm), and (*Z*)-methyl in DMAPP (1.72 ppm).

from buffer and cosubstrates, and the protons at C4 in IPP were obscured by the signal from residual H_2O in the sample. A similar spectrum was obtained when DMAPP was the starting substrate. An equilibrium ratio of IPP to DMAPP of ~1:2.5 was determined by comparing the intensities of the signals at 2.40 (the methylene protons at C2 in IPP) and 5.45 ppm (the olefinic proton at C2 in DMAPP).

The isomerization reaction was run in ^2H_2O and analyzed by ^{1}H and ^{2}H NMR spectroscopy to determine the extent of incorporation and the location of deuterium in IPP and DMAPP. The equilibrium ratio of IPP and DMAPP was determined from proton-decoupled ^{31}P NMR spectra (data not shown) by comparing the intensities of the AB quartets for the phosphorus nuclei in the diphosphate moieties of each

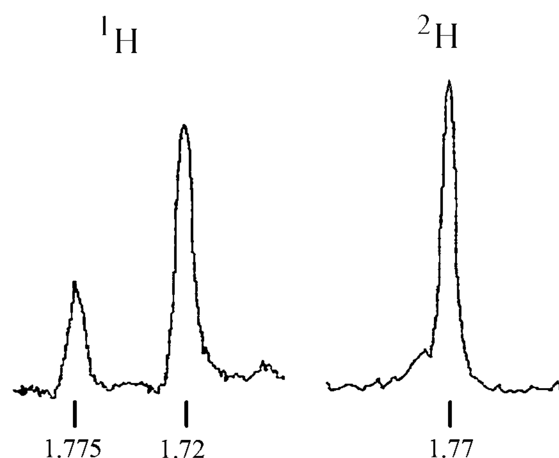


Figure 2. ^{1}H and ^{2}H NMR spectra of the reaction mixture after equilibration of DMAPP with type II IPP isomerase in ^2H_2O. Samples were prepared for NMR measurements in H_2O and analyzed by the procedures described in Figure 1.

compound. In addition, the relative amounts of IPP and DMAPP in the equilibrium mixtures were determined by gas chromatographic (GC) and gas chromatographic/mass spectrometric (GC/MS) analysis of isopentenol and dimethylallyl alcohol after hydrolysis of the diphosphates with alkaline phosphatase (see below). The values were similar to those found upon incubation in H_2O.

It was straightforward to determine the regiochemistry and stereochemistry of deuterium incorporation into the methyl groups in IPP and DMAPP by measuring the relative intensities of their ^{1}H and ^{2}H signals following isomerization in ^2H_2O. Figure 2 shows the methyl regions of ^{1}H and ^{2}H spectra for the equilibrium mixture starting with DMAPP. Similar results were obtained when starting with IPP (data not shown). The ^{1}H spectrum shows two peaks of unequal intensity for the (*Z*)-methyl in DMAPP at 1.72 ppm and the methyl in IPP at 1.775 ppm. There is no peak for the (*E*)-methyl in DMAPP. Correspondingly, the ^{2}H spectrum has a single peak for the (*E*)-methyl in DMAPP at 1.77 ppm. Thus, the hydrogens in the (*E*)-methyl of DMAPP were exchanged for deuterium during the multiple isomerizations of IPP and DMAPP required for the two compounds to reach equilibrium.

The relative intensities of signals for the methyl group in IPP at 1.775 ppm and the (*Z*)-methyl group in DMAPP at 1.72 ppm were similar to that seen when the isomerization was run in H_2O. A ^{2}H spectrum of the equilibrium mixture (Figure 3) showed two peaks, a broad singlet at 1.77 ppm for the (*E*)-methyl in DMAPP and a less intense broad singlet at 2.40 ppm for the deuterium at C2 in IPP. The intensity of the ^{1}H peak at 2.4 ppm for the C2 methylene in IPP suggests that only one of the two methylene hydrogen atoms exchanged with ^2H_2O during the isomerization. The peak at 2.40 ppm in the ^{2}H spectrum of the equilibrium mixture of IPP and DMAPP, coupled with the absence of ^{2}H incorporation at C2 of DMAPP, indicates that the installation/removal of the proton at C2 in both substrates is stereoselective. Thus,

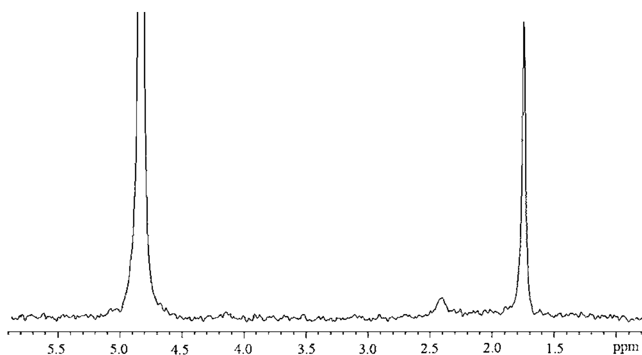


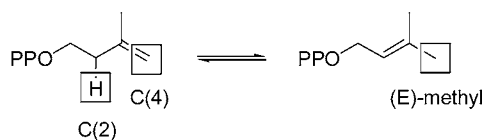
Figure 3. ^{2}H NMR spectrum of the reaction mixture after equilibration of IPP with type II IPP isomerase in ^2H_2O. Resonances are seen for the (*E*)-methyl in DMAPP (1.77 ppm) and the proton at C2 in IPP (2.40 ppm). Resonances for the C4 protons in IPP are obscured by deuterium in the solvent (~4.8 ppm).

the isomerization of IPP and DMAPP results in the incorporation of protons from water into the (*E*)-methyl group in DMAPP and one of the two prochiral hydrogens at C2 in IPP. No evidence was seen for replacement of protons by deuterium from ^2H_2O at the methyl group in IPP or the proton at C2 and the (*Z*)-methyl group in DMAPP.

Integration of the C4 methylene peak of IPP at ~4.86 ppm is compromised by trace amounts of water in the NMR samples, which gave a peak centered at 4.8 ppm. Evidence for incorporation of water protons at that position was provided by comparing GC mass spectra for isopentenol and dimethylallyl alcohol obtained from hydrolysis of IPP and DMAPP following equilibration in ^2H_2O with those from authentic samples of the alcohols.

The prominent peaks and the associated species in the mass spectrum of isopentenol are shown in Figure 4. The corresponding mass spectrum of isopentenol from IPP equilibrated by isomerase in ^2H_2O showed the following mass shifts corresponding to incorporation of deuterium. The molecular ion (M^+) and the fragment ion at *m/z* 71 were shifted to higher *m/z* (mass/charge) by 3 mass units. The single intense peak at *m/z* 68 for loss of water was shifted to give two peaks of almost equal intensity at *m/z* 70 (elimination of ^{2}H from C2) and *m/z* 71 (elimination of ^{1}H from C2). The McLafferty peak at *m/z* 56 was shifted to *m/z* 59, and the fragment ion at *m/z* 41 (cleavage of the C2−C3 bond) was shifted by two mass units to *m/z* 43. Taken together, these peaks demonstrate that isopentenol had three deuteriums, one

Scheme 2



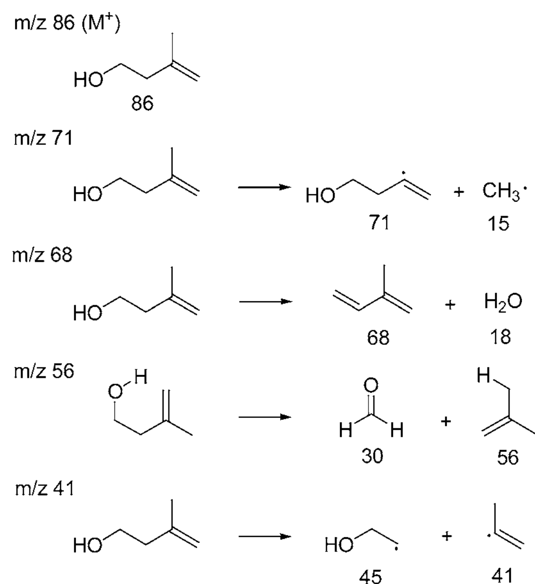


Figure 4. Prominent peaks in the mass spectrum of isopentenol. A 100 μL portion of the NMR sample was adjusted to pH 9.8 with 167 mM glycine/29 mM HEPES buffer containing 8 mM $MgCl_2$, 5 mM $ZnCl_2$, 3 mM DTT, and 20 units of calf alkaline phosphatase in a final volume of 400 μL. The mixtures were incubated for 1 h at 37 °C and then extracted with *tert*-butyl methyl ether. A portion of the extract was analyzed by gas chromatography on a 30 m × 0.25 μm DBS capillary column (J & W Scientific) with a temperature gradient from 70 to 150 °C at 2 °C/min with detection by flame ionization or by electron impact mass spectrometry at 70 eV.

at C2 and two at C4. The mass spectrum of dimethylallyl alcohol from the same sample had a molecular ion at *m/z* 89 and prominent fragment ions at *m/z* 71 (loss of C^2H_3) and *m/z* 74 (loss of C^1H_3). No peaks were seen above the molecular ion indicative of incorporation of additional deuterium into IPP or DMAPP.

Our results confirm that protons from water are incorporated into IPP and DMAPP during the isomerization catalyzed by type II IPP isomerase as shown in Scheme 2).[7] Even after prolonged exposure of IPP and DMAPP to type II IPP isomerase in ^2H_2O, NMR and mass spectral data indicated that only the hydrogens in the (*E*)-methyl group of DMAPP, one of the two prochiral hydrogens at C2 in IPP, and the hydrogens at C4 in IPP were replaced by deuterium. Under similar conditions with the type I enzyme from yeast, only the protons at C1 in IPP and DMAPP remained as a result of a slower exchange of H_a at C2 of IPP, the methyl protons in IPP, and the (*Z*)-methyl protons in DMAPP. Thus, it appears that isomerization of IPP and DMAPP by the type II enzyme is more stereoselective than that by its type I counterpart.

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