Enzyme Mechanisms

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Mechanistic Studies of an IspH-Catalyzed Reaction: Implications for Substrate Binding and Protonation in the Biosynthesis of Isoprenoids**

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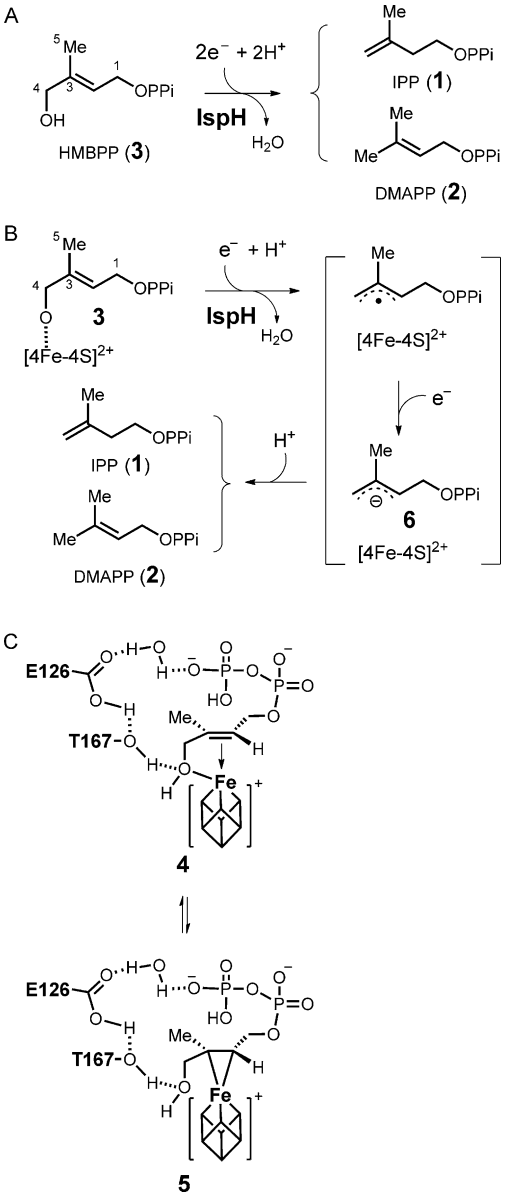
Isoprenoids are found widely in nature and have remarkably diverse structures.[1] They are utilized by all living organisms for a variety of biological functions, including serving as structural components of cell membranes, key constituents of electron-transport chains, and hormones to regulate various physiological processes.[2] Many isoprenoids, produced as secondary metabolites, function as defense agents for the producers and have been a rich source for human medicines.[2,3]

Successive condensation of isopentenyl diphosphate (IPP, **1**, Scheme 1) and dimethylallyl diphosphate (DMAPP, **2**) to construct an isoprenyl backbone of desired length is a common step in the biosynthesis of all isoprenoids.[1a,4] For decades, it was believed that the mevalonic acid (MVA) pathway is the sole source of IPP and DMAPP in all organisms.[5] Only recently, a second pathway, the deoxyxylulose phosphate (DXP) pathway (also known as the methyl erythritol phosphate (MEP) pathway) was discovered,[1b,c,6] in which both IPP and DMAPP are coproduced from 4-hydroxy-3-methyl-2-butenyl diphosphate (HMBPP, **3**) catalyzed by the IspH enzyme (Scheme 1 A).[7–11] Since IspH is not present in humans, and isoprenoids are essential for the survival of many pathogenic microorganisms, IspH has become an attractive target for the development of new antimicrobial drugs.[12]

The IspH-catalyzed conversion of **3** to **1** and **2** is an overall two-electron reductive dehydroxylation reaction. Previous biochemical, spectroscopic, and structural studies of IspH revealed the presence of a [4Fe-4S] cluster having a unique iron site to which the 4-hydroxy group of HMBPP (**3**) is anchored (see **4** in Scheme 1 C).[10b] This iron–sulfur cluster plays an essential role in electron transfer during IspH catalysis.[8,13] A mechanism resembling that of Birch reduction has been proposed for the IspH-catalyzed reaction (Scheme 1 B).[8a,b,9,13] However, in view of the close proximity of the C2–C3 double bond of **3** to the unique iron site of the [4Fe-4S] cluster (roughly 2.9–3.0 Å) in the crystal structure of the IspH–HMBPP complex (Figure 1) and the results of ENDOR studies of an IspH E126A mutant,[10] an alternative mechanism involving η^2 coordination between the C2–C3 double bond and the reduced $[4Fe\text{-}4S]^+$ cluster (see **5** in Scheme 1 C) was also proposed.[8c] To further investigate the mechanism of



Scheme 1. A) The IspH-catalyzed C4-dehydroxylation reaction, B) a possible mechanism of IspH-catalyzed reaction, and C) two models of the one-electron-reduced IspH–HMBPP complex. PPi = $P_2O_6^{3-}$.

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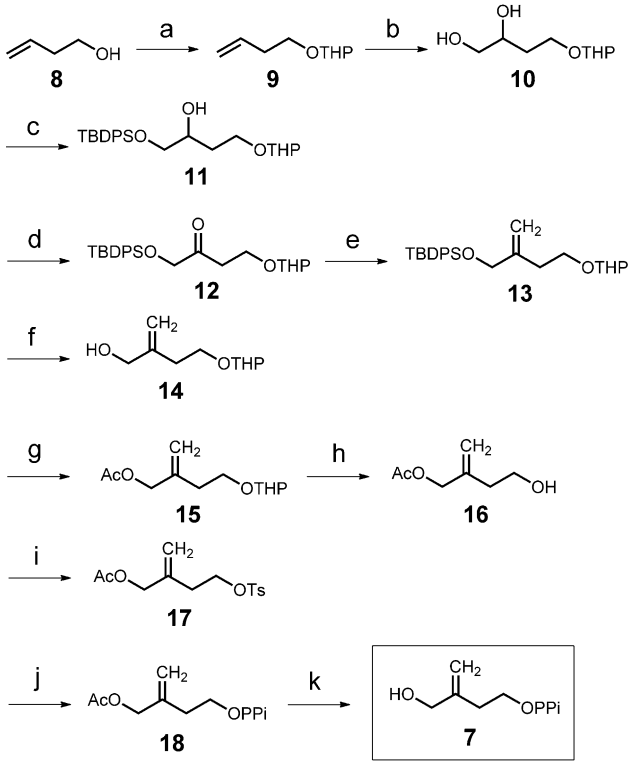
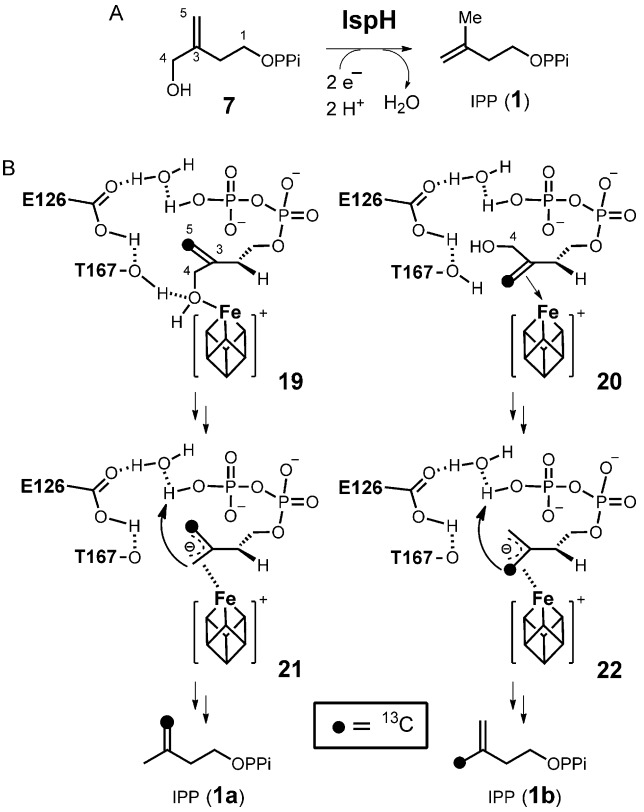
this intriguing reaction, we prepared a substrate analogue, 3-(hydroxymethyl)but-3-en-1-yl diphosphate (**7**, Scheme 2), which is expected to bind to the $[4Fe\text{-}4S]^{+}$ cluster in two different orientations (see **19** and **20** in Scheme 3) depending on whether formation of an metallacycle intermediate is part of the catalysis. We report herein on the experimental details and the mechanistic implications of these studies. The evaluation of the competence of **7** as an IspH substrate and analysis of the protonation of the allylic anion intermediate (**6**) shed new light on the mode of action of IspH.



Scheme 2. Reagents and conditions: a) DHP (1.20 equiv), CH_2Cl_2, 0 °C, 2 h, 95%; b) OsO_4 cat., NMO (1.50 equiv), acetone/potassium phosphate (KPi) buffer (100 mM, pH 7.40)/THF 2:2:1, RT, 3 h, 90%; c) TBDPSCl (1.10 equiv), imidazole (2.00 equiv), DMAP cat., CH_2Cl_2, RT, 12 h, 83%; d) DMSO (3.00 equiv), $(COCl)_2$ (1.50 equiv), Et_3N (5.00 equiv), CH_2Cl_2, −78 °C to RT, 1 h, 85%; e) PPh_3CH_3I (2.00 equiv), *n*BuLi (1.90 equiv), THF, 0 °C to RT, 2 h, 80%; f) TBAF (2.00 equiv), THF, 90%; g) Ac_2O (4.00 equiv), pyridine, RT, 14 h, 91%; h) AcOH/H_2O/THF 3:3:1, RT to 50 °C, 5 h, 80%; i) TsCl (2.00 equiv), pyridine, 0 °C, 12 h, 90%; j) $[N(nBu)_4]_3P_2O_7H$ (1.30 equiv), MeCN, RT, 5 h, 40%; k) NaOH (2.50 equiv), 0 °C to RT, 48 h, 60%. NMO = 4-methylmorpholine *N*-oxide, TBDPS = *tert*-butyldiphenylsilyl, DMAP = 4-dimethylaminopyridine, TBAF = tetrabutylammonium fluoride, Ts = 4-toluenesulfonyl.

The synthesis of **7** followed the reaction sequence delineated in Scheme 2 (see the Supporting Information for details). The capability of IspH to process **7** as a substrate was determined by monitoring the progress of the reaction with ^{1}H NMR spectroscopy.[8b, 9b] The only turnover product found in the incubation is IPP (**1**, Scheme 3 A), which was isolated and verified by ^{1}H NMR spectroscopy and high-resolution mass spectrometry. The kinetic parameters for the conversion



Scheme 3. A) The conversion of 3-(hydroxymethyl)but-3-en-1-yl diphosphate (**7**) to IPP (**1**) by IspH. B) Two possible binding modes of [5-^{13}C]-**7** in the active site of IspH, and the anticipated respective outcomes of the pronotation step.

of **7** to **1** by IspH were measured using the methyl viologen assay.[8b] The analysis yielded a k_{cat} of $(484 \pm 6.5)\ min^{-1}$ and a K_m for **7** of $(694 \pm 79)\ \mu M$. The k_{cat} value is comparable to that of $(604 \pm 17)\ min^{-1}$ determined for the native substrate HMBPP (**3**) under similar conditions. However, the K_m of **7** is nearly 35-fold higher than that of **3**, resulting in a 44-fold reduction of the catalytic efficiency (k_{cat}/K_m) relative to that of HMBPP (**3**). Although **7** is a poor substrate, this result nevertheless demonstrates that the substrate of IspH does not necessarily have to have a double bond in the middle of its carbon skeleton as in **3**. This finding challenges the proposed metallacycle model since the olefin moiety in **7** is further away from the apical iron atom of the [4Fe-4S] cluster (see **19** in Scheme 3 B) if the binding mode is the same as that observed in the recent IspH–HMBPP complex.[10b]

The fact that IPP is the sole product of the reaction of **7** and IspH is clearly different from the reductive dehydroxylation of **3** by IspH in which both IPP (**1**) and DMAPP (**2**) are produced in a ratio of approximately 5:1.[7d, 9b] This ratio is different from the roughly 1:3 distribution of IPP and DMAPP at thermodynamic equilibrium.[8a] The production of both IPP and DMAPP from HMBPP by IspH may be explained by the specific binding mode of **3** in the active site of IspH.[10b] As shown by the crystal structure in Figure 1, HMBPP (**3**) binds to IspH in a bent conformation with its 4-OH group coordinated to the apical iron of the [4Fe-4S] cluster and its five-carbon backbone sandwiched between the

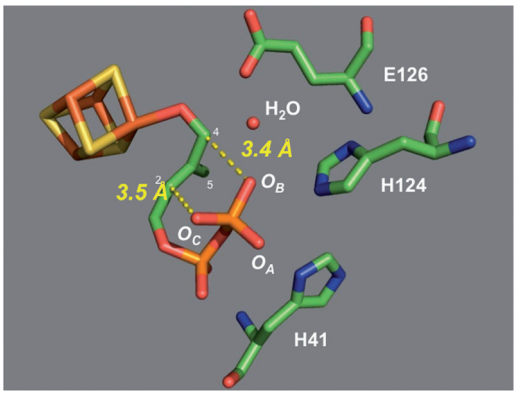


Figure 1. The active site of IspH with the 4-OH group of HMBPP (**3**) bound to the [4Fe-4S] cluster. The distances between O_B to C4 and O_c to C2 are roughly 3.4 and 3.5 Å, respectively (pdb code: 3KE8).

[4Fe-4S] cluster and the C1-pyrophosphate group in the enzyme active site. With such geometric constraints and the lack of a nearby proton source, it was proposed that the terminal phosphate group of HMBPP serves as the proton donor in the final step (**6**→**1** and **2**, in Scheme 1B) of the dehydroxylation reaction,[8a,9b,10b] where the negative charge of the proposed allylic anion intermediate (**6**) is delocalized through C2, C3, and C4. Because O_C and O_B (see Figure 1) are within 3.4–3.5 Å of the C2 and C4 positions of HMBPP, they are likely involved in the protonation at C2 and C4 to yield IPP and DMAPP, respectively. This hypothesis is consistent with the *pro*-S stereochemistry observed for the C2-protonation step (to form IPP from HMBPP).[14] Unlike O_C, O_B forms a hydrogen bond with a water molecule, which is also within hydrogen-bonding distance to E126. Thus, the ratio of IPP and DMAPP may simply reflect the different protonation state of O_B and O_C in the enzyme–substrate complex. Although the water molecule generated in the dehydroxylation step may serve as an alternative proton source at C4, the fact that incubation with HMBPP and its monofluoro analogue afforded IPP and DMAPP in the same ratio (approximately 5:1)[9b] is most consistent with the pyrophosphate (or the water molecule between O_B and E126) serving as the proton source (see **21**/**22**).

When compound **7** is used as the substrate, the negative charge of the proposed allylic anion intermediate will be delocalized among C3, C4, and C5 (**21**/**22** in Scheme 3B) instead of C2, C3, and C4 (**6**), as seen in HMBPP (**3**). Hence, owing to the proximal location of O_B to C3, C4, and C5, O_B is most likely the proton donor and protonation at either C4 or C5 will yield IPP (**1**) as the sole product, consistent with the experimental observations. However, since O_B is located closer to C4 (3.4 Å) than to C5 (4.6 Å), protonation is expected to occur largely at C4. Taking advantage of the anticipated preferential protonation at the site closer to O_B, we probed this process using [5-^{13}C]-**7**. We anticipated that if coordination of the 4-OH group of **7** to the [4Fe-4S] cluster is the anchor that positions the substrate in the enzyme active site (shown as **19** in Scheme 3B), protonation at C4 of the allylic anion intermediate (**21**) would yield **1a** when labeled **7** is used as the substrate. In contrast, if the reaction proceeds via an η^2-alkenyl intermediate, as proposed by the metallocycle mechanism, coordination of the double bond of **7** to the iron–sulfur cluster may be a prerequisite to substrate orientation in the active site (Scheme 1C). Consequently, [5-^{13}C]-**7** would bind to IspH in a conformation represented by **20**. Subsequent protonation of **22** at the carbon closer to O_B (now C5) should afford **1b** as the product.

The labeled substrate [5-^{13}C]-**7** was synthesized according to the reaction sequence shown in Scheme 2, except [^{13}C]-PPh_3CH_3I was used in the conversion of **12** to **13** (see the Supporting Information for details). The [5-^{13}C]-labeled product was incubated with IspH, and the reaction was quenched at appropriate time intervals (60% and 100% conversion). After IspH was removed, the incubation mixture was analyzed by ^{13}C NMR spectroscopy. As shown in Figure 2A, [^{13}C]-**7** by itself gives one enriched ^{13}C signal at 111.6 ppm. When the reaction was run to completion (Figure 2B), only one product was obtained. The sole signal that

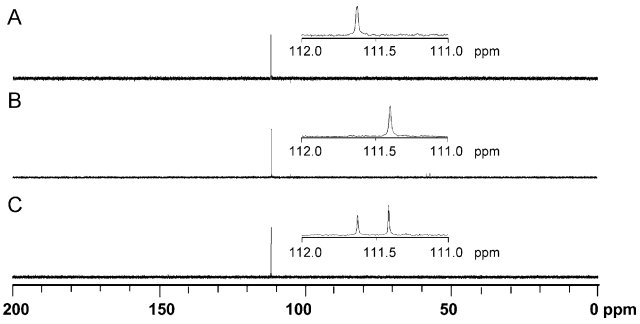


Figure 2. ^{13}C NMR analysis of the incubation of [5-^{13}C]-labeled **7** (5.0 mM) with IspH in 100 mM NaPi, pH 8.0 at 37°C: A) in the absence of enzyme; B) reaction was run with 5.0 μM IspH to completion (quenched after incubation for 1 h); C) reaction was run to 60% completion with 1.0 μM IspH (quenched after incubation for 30 min).

appears at 111.4 ppm can be assigned to the resonance of the terminal methylene carbon of the [^{13}C]-labeled IPP product (**1a**). When the reaction was quenched at 60% conversion (Figure 2C), signals for both labeled **7** and **1a** were present. Interestingly, there were no signals corresponding to [^{13}C]-**1b**, which should have an enriched signal in the region of approximately 25 ppm (i.e., the chemical shift for the IPP methyl group). These results are consistent with the proposal that coordination of the 4-OH group to the apical iron site is important in positioning the substrate for reaction with the [4Fe-4S] cluster, and C4 of **7** is the protonation site in the IspH-catalyzed dehydroxylation of **7** to give **1a**.

These results are significant for two reasons. First, the outcome of the protonation experiments with [5-^{13}C]-**7** (i.e., only **1a** is produced from **7**) provide evidence supporting a catalytic role for the terminal phosphate group of the substrate in the final protonation step of the IspH reaction. Second, our data also shed new light on the interaction between the substrate double bond and the [4Fe-4S] cluster, which has been proposed to play an important role in IspH catalysis.[8c] However, the precise nature of this interaction has been controversial: it may be a transannular effect

contributing to substrate binding as suggested by Shanmugam et al.,[15] or the driving force to form a metallacycle intermediate as proposed by Wang et al. (Scheme 1C).[8c] By comparing the incubation results with **3** and **7**, it is now clear that while the olefin moiety is important for substrate binding and turnover, the formation of a metallacycle between the double bond and the unique iron site of the [4Fe-4S] cluster is not a prerequisite for catalysis. Since the key coordinating ligand to the iron–sulfur cluster has now been shown to be the 4-OH group rather than the olefinic π system of substrate **7** (Scheme 3B), the proposed metallacycle mechanism is less likely than the Birth reduction type mechanism (at least in the conversion of **7** to **1**).[8c] Clearly, more studies are required to further delineate the catalytic mechanism of IspH. Additional experiments are also needed to determine how the reaction flux (IPP versus DMAPP) is controlled in the IspH reaction because this distribution is crucial for cellular survival. Efforts on both fronts are in progress.

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