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Study of IspH, a Key Enzyme in the Methylerythritol Phosphate Pathway Using Fluoro-Substituted Substrate Analogues

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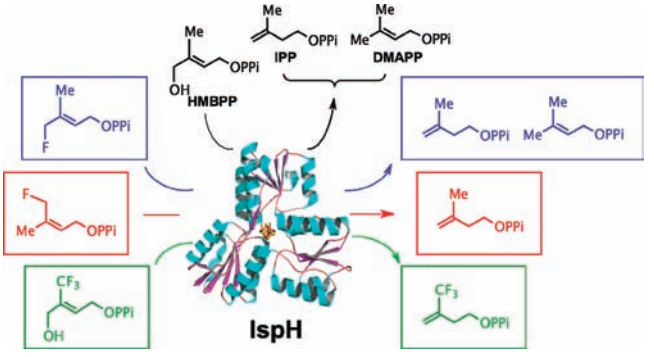
ABSTRACT



IspH, a [4Fe-4S]-cluster-containing enzyme, catalyzes the reductive dehydroxylation of 4-hydroxy-3-methyl-butenyl diphosphate (HMBPP) to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) in the methylerythritol phosphate pathway. Studies of IspH using fluoro-substituted substrate analogues to dissect the contributions of several factors to IspH catalysis, including the coordination of the HMBPP C_4–OH group to the iron–sulfur cluster, the H-bonding network in the active site, and the electronic properties of the substrates, are reported.

IspH is a [4Fe-4S]-cluster-containing enzyme found in the newly discovered isoprene biosynthetic pathway known as the methylerythritol phosphate (MEP) pathway.[1] It catalyzes the last step of the MEP pathway by reductive dehydroxylation of the C_4–OH group of 4-hydroxy-3-methyl-butenyl diphosphate (HMBPP, **1**) to isopentenyl diphosphate (IPP, **2**) and dimethylallyl diphosphate (DMAPP, **3**, Figure 1), which are the two building blocks for all isoprenoids.[2,3] While the MEP pathway is not present in humans, it is essential for many pathogenic microorganisms. Therefore, the enzymes involved in this pathway are attractive new targets for antimicrobial drugs.[3,4] Early results showed that its labile [4Fe-4S] cluster contains a unique iron site,[5−10] to which the C_4–OH group of HMBPP binds (**4** in Scheme 1).[6,7,11] The bound HMBPP adopts a hairpin conformation in the active

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site with the carbon chain skeleton sandwiched between its pyrophosphate group and the [4Fe-4S] cluster (Figure 1).[12] Due to the lack of a nearby proton source, protonation of the allylic anion intermediate (**8**, Scheme 1) during turnover has been proposed to be mediated by the terminal phosphate group of HMBPP.[8,11,12]

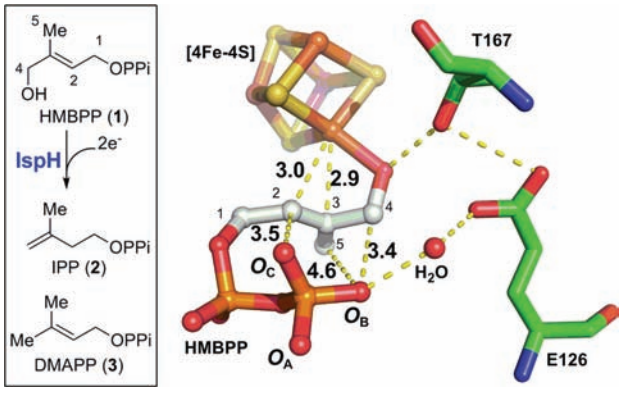
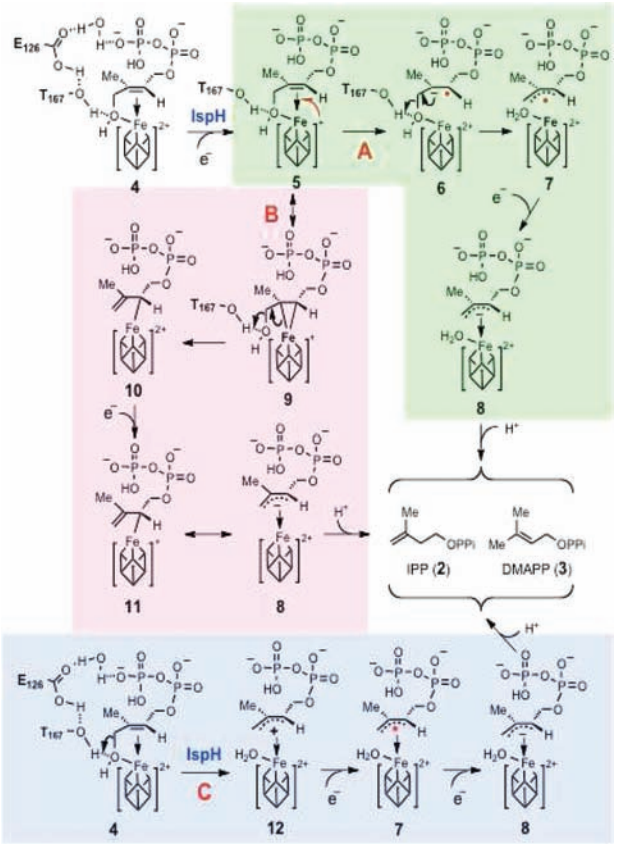


Figure 1. Reaction catalyzed by IspH, and the IspH-HMBPP (**1**) complex in the active site. Due to the short distance (~ 2.9–3.0 Å) between the olefinic moiety (C_2–C_3) of **1** and the unique apical iron site, some interactions between the double bond of **1** and the iron–sulfur cluster may exist.

Studies in recent years have led to three mechanistic models for IspH catalysis.[5,8,11,13–16] Mechanism A proposed by Rohdich et al. (Scheme 1, route A) resembles a Birch reduction in which a one-electron transfer from the reduced iron–sulfur cluster to HMBPP (**5** → **6**) triggers the C_4-deoxygenation from the nascent radical anion species (**6**) to form an allyl radical intermediate (**7**).[11,14,15] Subsequent one-electron reduction (**7** → **8**) followed by protonation produces IPP (**2**) or DMAPP (**3**) depending on whether protonation occurs at C_2 or C_4 of **8**. Mechanism B proposed by Wang et al. (Scheme 1, route B) is based mainly on the biophysical characterization of a paramagnetic species trapped in the IspH E126A mutant.[17] In this organometallic model, formation of a substrate/metal π complex or an η^2-alkenyl/metallacycle (**9**) followed by dehydration gives an η^1-allyl intermediate (**10**). Upon the second one-electron transfer, **10** is reduced to an η^3-allyl complex (**11**), which is then protonated to form IPP or DMAPP. Alternatively, in mechanism C (Scheme 1, route C) proposed by Altincicek et al.,[13] the Lewis acidity of the metallo-center may facilitate the heterolytic cleavage of the C_4–OH bond to form an allylic cation intermediate (**12**), which then undergoes two-electron reduction and protonation to yield the final products. Although coordination of the C_4–OH group of HMBPP to the [4Fe–4S] cluster is important for all three proposed mechanisms, formation of metallocycle intermediates using the HMBPP olefinic moiety is specific for mechanism B, and the involvement of a cation intermediate is unique for mechanism C.

Scheme 1. Proposed Mechanisms for IspH-Catalyzed Reaction



To learn more about the substrate specificity and to assess the energetic contributions of various interactions in the active site during IspH catalysis, a series of fluoro-substituted substrate analogues were synthesized and their competence as IspH substrates was examined (Scheme 2).[18] The results and insights gained from these studies are reported herein.

Previously, we reported that IspH can process the monofluoro analogue **13** to produce both IPP (**2**) and DMAPP (**3**) in a ratio of ~5:1.[11] The kinetics of **13** as an

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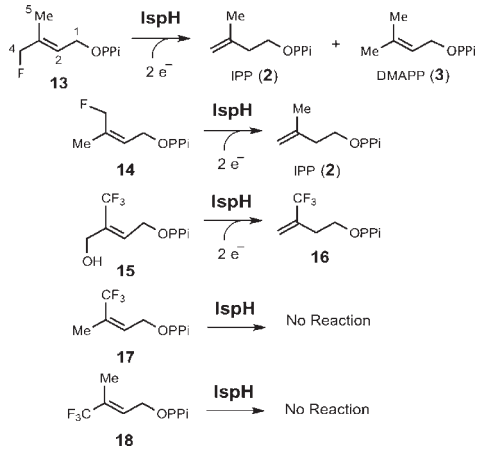
(18) Syntheses of the substrate analogues listed in Scheme 2 are included in the Supporting Information. These compounds were evaluated as IspH substrates by a recently developed assay.[7] After incubation of substrate analogues with IspH, the reaction mixture was purified by HPLC, and the turnover products were isolated and characterized by ^{1}H NMR and high-resolution mass spectrometry. The identities of these products were further confirmed by comparison with synthetic standards (see Supporting Information).

Scheme 2. Products Produced from Incubations of Substrate Analogues with IspH[a]



[a] Refer to Figure 1 for the distances between various atoms.

IspH substrate was re-examined in this work by a more sensitive assay using methyl viologen, instead of flavodoxin-flavodoxin reductase, as the electron mediator.[7] In the new assay, the IspH activity is nearly 100-fold higher than that of the NADPH-flavodoxin-flavodoxin reductase system.[11] As listed in Table 1, the K_m for **13** is 5.3-fold higher and k_{cat} is 21.8-fold lower with a total of ~115-fold reduction of k_{cat}/K_m relative to that of HMBPP (**1**). Clearly, replacing the C_4−OH group by a fluorine substituent affects overall catalysis. The reduction in catalysis may result from a combination of several factors. First, substitution of the C_4−OH group by a fluorine atom will dramatically reduce the interaction between the substrate and the [4Fe−4S] cluster apical iron site because fluorine is a poor metal ligand. Second, the electronegativity difference between a fluoro and a hydroxyl group will change the electronic properties of the double bond, which, in turn, may affect IspH activity. Third, the HMBPP C_4 hydroxyl group is part of the H-bonding network shown in Figure 1. To dissect the relative contributions of the above factors to IspH catalysis, four additional substrate analogues, **14**, **15**, **17**, and **18**, were prepared and examined (Scheme 3 and Table 1).

The substrate analogue, **14**, has the fluorine group at the C_5 position instead of C_4. Such a change should have little impact on the electronic properties of the olefin but will affect the hydrogen-bond network with the active site residues T167 and E126. Thus, a comparison with analogue **13** may allow us to better assess the effect of the hydrogen-bonding network in IspH catalysis. As shown in Table 1, incubation of **14** with IspH showed that IspH can accept **14** as a substrate and produce IPP (**2**) as the sole product. In comparison with HMBPP (**1**), the K_m for **14** is 25-fold higher, the k_{cat} is 72-fold lower, and the k_{cat}/K_m is ~1783-fold reduced. The significant reduction in k_{cat}/K_m for **14** relative to **1** is not surprising and can be attributed to a combination of the lack of direct coordination of **14** to the apical iron site of the iron−sulfur cluster and the change in the electrophilic nature of the double bond. Notably, comparing **13** with **14**, the K_m for **14** is 4.7-fold higher, the k_{cat} is 3.3-fold lower, and the k_{cat}/K_m is ~15.5-fold reduced. The observed activity differences between **13** and **14** suggest that the C_4-fluoro group of **13** may still maintain partial hydrogen-bond interaction with the T167 and E126 side chain rendering it a better substrate for IspH than **14**.

To further probe the effects of the substrate electronic properties on IspH catalysis, (*Z*)-4,4,4-trifluoro-3-(hydroxymethyl)-but-2-en-1-yl diphosphate (**15**) was prepared. The strong electron-withdrawing nature of the trifluoromethyl substituent will destabilize the proposed allylic carbocation intermediate (**12**) which will in turn reduce the reaction rate to a great extent if mechanism C is operative. When **15** was incubated with IspH, 3-(trifluoromethyl)but-3-en-1-yl diphosphate (**16**) was produced as the only product (see Scheme 3).[18] Clearly, IspH is capable of catalyzing the dehydroxylation of **15**, but not the subsequent dehalogenation reaction. A similar outcome was also observed when (*Z*)-4,4,4-trifluoro-3-methyl-but-2-en-1-yl diphosphate (**17**) or (*E*)-4,4,4-trifluoro-3-methyl-but-2-en-1-yl diphosphate (**18**) was incubated with IspH and no product could be detected (Scheme 2). Although the k_{cat}/K_m value is reduced ~150-fold for **15** (compared to **1**), the k_{cat} is only 6.6-fold lower. These data suggest that route C is less likely as the mechanism for IspH. A similar conclusion was also reached in a recent report in which incubation of IspH with several proposed carbocationic intermediates caused little inhibition against IspH.[19] The fact that the k_{cat} for **15** is ~3.3-fold greater than that of the monofluoro analogue **13** and ~10.9-fold higher than that of the monofluoro analogue **14** again underscores the important role played by the C_4 hydroxyl group in IspH catalysis.

Table 1. Summary of Activity Using Various Mechanistic Probes

probes	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\cdot min^{-1}$)
1	604 ± 17	19.7 ± 2.4	30.6
13	27.7 ± 2.2	104 ± 31	0.27
14	8.4 ± 1.9	489 ± 170	0.017
15	91.6 ± 2.2	447 ± 32	0.21
17	No detectable activity		
18	No detectable activity		

The fact that only IPP (**2**) is produced in the reaction with **14** is intriguing since both IPP (**2**) and DMAPP (**3**) are formed when HMBPP (**1**) is used as the substrate. As shown in Scheme 3A, generation of **2** and **3** from **1** results from protonation of the proposed allylic anion intermediate **8** at C_2 and C_4, respectively. The proton donor is

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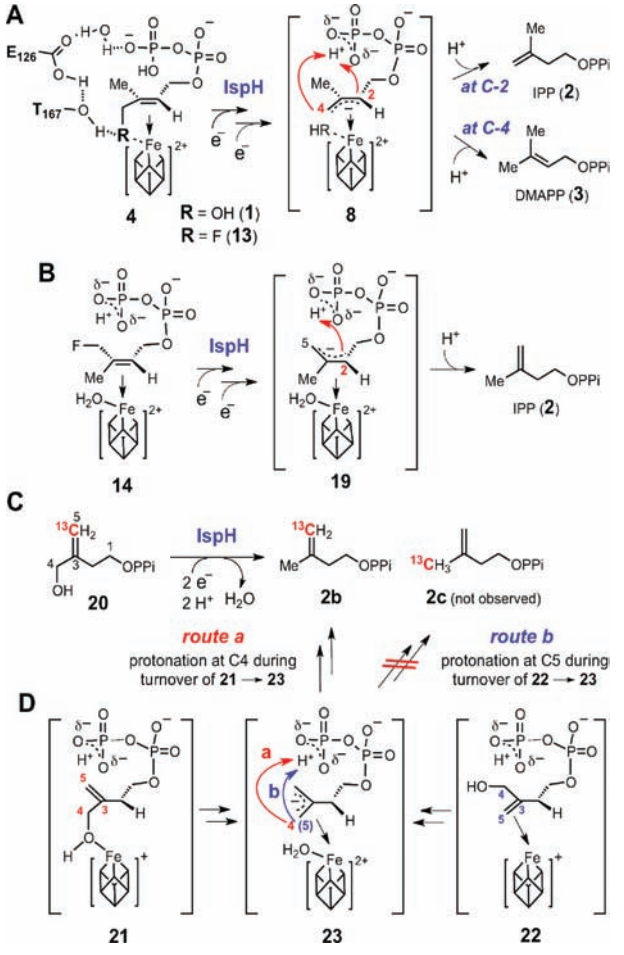
believed to be the terminal phosphate group of HMBPP, which is within ~3.4−3.5 Å of the C_2 and C_4 position of HMBPP (see Figure 1).[12,20] In the case of **14**, the negative charge of the proposed allylic anion intermediate (e.g., **19**, Scheme 3B) is delocalized through C_2, C_3, and C_5 instead of C_2, C_3, and C_4 as shown in intermediate **8** (see Scheme 3A). The fact that protonation takes place only at C_2, but not at C_5, in **19** likely reflects the distance and unfavorable orientation of C_5 of **19** relative to the phosphate group (e.g., O_B) in the active site (e.g., ~4.6 Å between C_5 and O_B, Figure 1).

Scheme 3. Mode of Protonation of the Allylic Anion Intermediate Generated during Turnover



The conclusions reached based on the outcomes of these fluoro-analogues are also consistent with that derived from the incubation of IspH with a [^{13}C]-labeled substrate analogue, 3-(hydroxymethyl)but-3-en-1-yl diphosphate ([5-^{13}C]-**20**, Scheme 3C),[21] which is expected to bind to the $[4Fe-4S]^+$ cluster in two different orientations depending on whether the reaction follows mechanism A or B. If the C_4−OH of **20** is the anchor that positions the substrate in the active site (**21** in Scheme 3D), protonation mediated by O_B at C_4 of the allylic anion intermediate (**23**, Scheme 3D, route a) would yield [^{13}C]-IPP (**2b**) as the product (also see Figure 1). In contrast, if the reaction proceeds via an η^2-alkenyl intermediate (**22** in Scheme 3D), protonation of the allylic anion at the carbon closer to O_B (now C_5, Scheme 3D, route b) should afford [^{13}C]-IPP (**2c**). The observation that **2b** is the sole product after incubation strongly suggests that the C_4−OH group, rather than the olefinic π-system, plays the dominant role in orienting the substrate in the active site and, thus, favors the Birch reduction model. Together, these observations strongly indicate that protonation of the allylic anion intermediate (**8**, **19**, or **23**) occurs at C_2 and/or C_4, but not at C_5.

In conclusion, the results obtained from these studies provide important insights into the mode of substrate binding and the mechanism of IspH. First, in view of the relatively minor influence of electron-withdrawing substituents on the reaction rate (e.g., **15**), a mechanism involving a carbocationic intermediate/transition state is less likely. Second, coordination of the C_4−OH group of HMBPP to the unique iron site of the [4Fe−4S] cluster and its involvement in the hydrogen-bond network (e.g., with T167 and E126) is crucial for efficient catalysis (**13**/**14** versus **1**, and **15** versus **17**). The adverse effects (both steric and electronic) imposed by a trifluoromethyl substituent in **17** can be balanced by the introduction of a C_4−OH group (**15** versus **17**). Third, protonation of the allylic anion intermediate is regiospecific and takes place only at C_2 and/or C_4 (see **8** → **2**/**3**, **19** → **2**, and **23** → **2b**), but not at C_5 (Scheme 3). These findings support the assigned role of the terminal phosphate group of HMBPP in mediating the final protonation step due to its close proximity to the C_2 and C_4 positions of the allylic anion intermediate (**8**, Schemes 1 and 3A). More experiments are in progress to further investigate this interesting example of an enzymatic reductive dehydroxylation reaction.

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Supporting Information Available. Details regarding experimental procedures, NMR characterization of the synthetic products as well as the incubation reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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