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J. Am. Chem. Soc., **2008**, 130 (7), 2164-2165 • DOI: 10.1021/ja710245d

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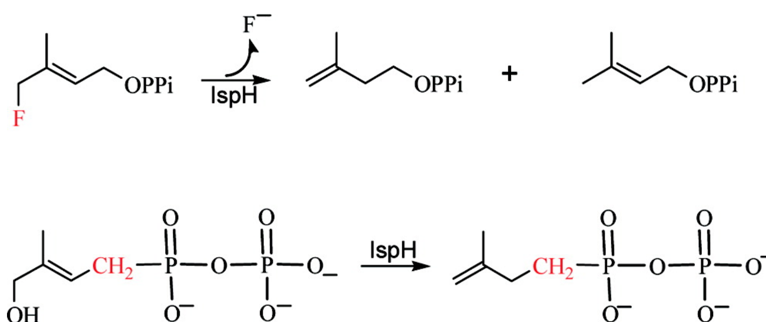

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Published on Web 01/25/2008

Mechanistic Studies of IspH in the Deoxyxylulose Phosphate Pathway: Heterolytic C−O Bond Cleavage at C_4 Position

Youli Xiao,† Zongbao K. Zhao,‡ and Pinghua Liu*,†

Department of Chemistry, Boston University, Boston, Massachusetts 02215, and Dalian Institute of Chemical Physics, CAS, Dalian 116023, P.R. China

Received November 16, 2007; E-mail: pinghua@bu.edu

Isoprenoids are one of the largest and most structurally diverse groups of metabolites in nature. Plants alone produce more than 30,000 isoprenoids.[1] The biosynthesis of isoprenoids requires two precursors, isopentenyl diphosphate (IPP, **2**) and its isomer, dimethylallyl diphosphate (DMAPP, **3**, Scheme 1). There are two different pathways to synthesize **2** and **3**: the deoxyxylulose phosphate (DXP) pathway and the mevalonic acid (MVA) pathway.[2] More importantly, these two pathways have a well-defined distribution among different kingdoms. Most pathogenic bacteria and protozoan parasites utilize the DXP pathway, while animals synthesize their isoprenoid precursors from acetyl-CoA via the MVA pathway. Plants have both DXP and MVA pathways.[2] Thus, mechanistic studies on the DXP pathway enzymes may lead to the development of mechanism-based inhibitors as herbicides, broad-spectrum antibiotics, and antimalaria drugs.[3,4]

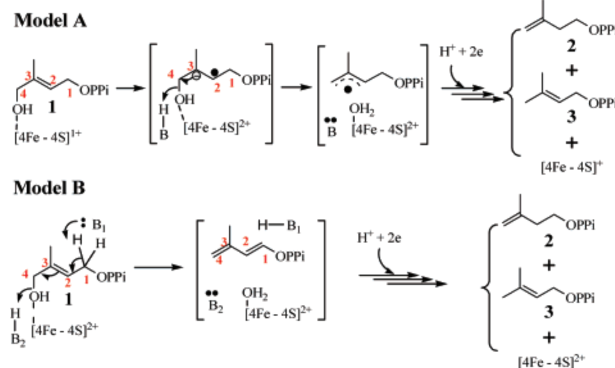
IspH in the DXP pathway catalyzes the reductive dehydration of (*E*)-4-hydroxy-3-methyl-2-butenyl diphosphate (HMBPP, **1**) to form **2** and **3**. Studies in the past few years led to the establishment of the in vitro *Escherichia coli* IspH catalytic system, which includes IspH, flavodoxin, and flavodoxin reductase.[5−12] The iron−sulfur cluster containing *E. coli* IspH, a 36 kDa protein, has only three conserved Cys residues (C12, C96, and C197).[11] Replacement of any of the three conserved cysteine residues reduced the catalytic activity by a factor of more than 70,000.[11] Recent EPR studies suggest that the isolated IspH has a $[3Fe-4S]^+$ cluster, which is attributed to the loss of the unique iron-site iron from a [4Fe−4S] cluster.[7, 10−11] In this work, we studied the IspH-catalyzed C−O bond cleavage mechanism. The activities at both C_1 and C_4 positions are examined.

Recently, Rodich et al. proposed a mechanism (model A in Scheme 1) to explain this unusual transformation.[7] In this model, it is believed that conformation restrictions at the enzyme active site favor the C_4 hydroxyl group as the leaving group instead of the better leaving group, pyrophosphate at the C_1 position.[13] In addition, the C_4 hydroxyl group is directly ligated to the unique iron site of the iron−sulfur cluster to facilitate this process. In this model, the iron−sulfur cluster is involved in both the dehydration and reduction steps. To generate the proposed allylic radical intermediate, it goes through the reduction of the double bond to produce a radical anion intermediate, which is then used to mediate the dehydration reaction. The resulting allylic radical intermediate is then reduced to the products (**2** and **3**). Model A (Scheme 1) is not the only route to the reductive dehydration products, **2** and **3**. Another option (model B in Scheme 1) is through a deprotonation-mediated dehydration reaction as found in aconitase, followed by reductions.

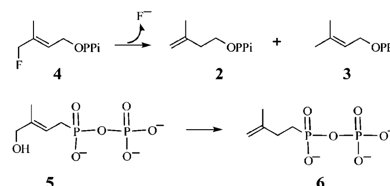
Two mechanistic probes, (*E*)-3-(fluoromethyl)-2-butenyl diphosphate (**4**) and (*E*)-4-hydroxy-3-methyl-2-butenyl pyrophosphonate

† Boston University.
‡ Dalian Institute of Chemical Physics.

Scheme 1. Two IspH Reaction Models



Scheme 2. Evaluation of Two IspH Substrate Analogues



(**5**) (Scheme 2), with a C−F or C−P linkage, respectively, are used to study the IspH-catalyzed C−O bond cleavage mechanism at both C_1 and C_4 positions. Our results clearly demonstrated that C_4 and not C_1 heterolytic C−O bond cleavage occurs and that the C_4 hydroxyl group is involved in substrate binding.

We have overproduced IspH in *E. coli* and purified the protein anaerobically to near homogeneity. The UV−vis spectrum of the anaerobically purified IspH is consistent with the presence of iron−sulfur clusters, e.g., the features at around 320 and 410 nm (Figure 1S). Under optimized conditions (Supporting Information), IspH protein with an A_{410}/A_{280} ratio of 0.4, close to that reported in literature, can be reproducibly obtained without reconstitution.[11] Purified IspH was characterized by two assays, the ^{1}H NMR assay to directly analyze the products, and the NADPH consumption assay at 340 nm to determine the kinetic parameters using NADPH-flavodoxin−flavodoxin reductase as a reducing system. The purified IspH has a k_{cat} of 11.6 min^{-1} and a K_m lower than 15 μM, which are close to the reported data.[11] The ratio of **2** to **3** produced from **1** was determined to be 5:1 (Figure 2S), which is similar to the reported 4.5:1 ratio.[11] For enzymatic transformation of pyrophosphate-containing substrates, divalent metals such as Ca^{2+} or Mg^{2+} are normally required. We examined the effect of divalent metals on IspH activity, and no significant effects were observed, which was consistent with that reported.[11]

Substrate analogue **4** (Scheme 2) with a fluorine atom replacing the C_4 hydroxyl group was synthesized according to a literature procedure.[14] Because of the strong electronegativity of the fluorine

10.1021/ja710245d CCC: $40.75 © 2008 American Chemical Society

atom, the C−F bond cleaves heterolytically. If the formation of the allylic radical in Scheme 1 is a one-step reductive homolytic C−O bond cleavage reaction, **4** will only be an inhibitor, and no enzymatic turnover is expected. Substrate analogue **5** (Scheme 2), with a pyrophosphonate function group (C−P bond) at the C_1 position, was also prepared (Scheme 1S). Because of the C−P bond stability, **5** will only be an inhibitor, and no enzymatic turnover will occur if C−O bond cleavage at the C_1 position is one of the steps in catalysis.

Interestingly, both **4** and **5** are IspH substrates (Scheme 2), with a k_{cat} of 0.55 min^{-1} and a K_m of 3.95 mM for **4** and a k_{cat} of 0.44 min^{-1} and a K_m lower than 15 μM for **5**. IspH converts **4** to a mixture of **2** and **3** in a ratio of 7:1, which is slightly higher than the ratio of products produced from **1**. The release of a fluoride anion as the product from **4** was directly detected using ^{19}F NMR (Figure 3S). ^{1}H NMR analysis of HPLC-purified product from **5** implies that only **6** is produced, and its identity was confirmed by comparison with the synthetic standards (Figures 4S and 5S).

The results from the above studies are mechanistically informative and provide the reactivity information at both C_1 and C_4 positions. Recent EPR studies suggest that IspH is a unique iron-site-containing [4Fe−4S] cluster protein.[11] However, the IspH-catalyzed reaction does not fall into any of the two well-studied categories of these unique proteins. The first class is the dehydratase family. The dehydration reactions catalyzed by this class of enzymes do not involve redox chemistry. Substrates coordinate to the $[4Fe-4S]^{2+}$ cluster at the unique iron site, and the $[4Fe-4S]^{2+}$ cluster serves as a Lewis acid to facilitate the heterolytic C−O bond cleavage.[15,16] Many examples of this class of enzyme are known, and one of the best characterized enzymes is aconitase.[16] The other class is the radical SAM superfamily members,[17−22] where SAM coordinates to the $[4Fe-4S]^{+}$ cluster at the unique iron site. One-electron transfer from $[4Fe-4S]^{+}$ to SAM leads to the production of an adenosyl radical, which is then used to mediate many energetically challenging reactions.[23−28]

For **4**, the K_m is increased by at least 200-fold compared to that of **1**, while the k_{cat} is only about 4.7% that of **1**. The K_m increase and k_{cat} decrease observed for **4** are all consistent with the known chemistry at the $[4Fe-4S]^{2+}$ cluster unique iron site, such as that of aconitase.[16] Therefore, the C_4 hydroxyl group of **1** is most likely a ligand of the $[4Fe-4S]^{2+}$ cluster unique iron site as it is in aconitase. Replacing the hydroxyl group by a fluorine atom, which is a weaker ligand, disrupts the interaction and causes a dramatic increase in K_m. Also, because the iron−sulfur cluster in the aconitase-type of enzymes serves as a Lewis acid to facilitate the dehydration process, the lack of this driving force in **4** explains the significantly lower activity (k_{cat}). In addition, because the strong electronegative nature of the fluorine atom precludes a homolytic C−F bond cleavage in **4**, the production of **2** and **3** from **4** suggests that the C_4 position C−O bond is heterolytically cleaved as suggested in Scheme 1.

The production of **6** from **5** is equally informative. It provides the direct evidence for supporting that no C−O bond scission occurs at the C_1 position during the reaction, as proposed in Scheme 1. The fact that the K_m value for **5** is very close to that of **1** suggested that a similar substrate-binding fashion is involved for both **1** and **5**. Interestingly, the k_{cat} for **5** decreases significantly (∼26-fold) compared to that of **1**, which implies that the C_1 position may be involved in the reaction as suggested in model B of Scheme 1. It is also possible that the reaction follows the model A mechanism, but the electron transfer or protonation steps are not optimal due to the shortage of one bridging oxygen in analogue **5** compared to substrate **1**.

In conclusion, IspH does not fall into the two known classes of unique iron-site-containing iron−sulfur proteins. Biochemical data reported herein provide crucial evidence to not only support the integrity of the C_1 position C−O bond during reaction, but also suggest a heterolytic C−O bond cleavage at the C_4 position for IspH-catalyzed reductive dehydration reaction. Also, the nearly 200-fold increase in K_m with **4** relative to **1** indicates that the C_4 hydroxyl group is involved in substrate binding. We are currently carrying out more in-depth investigations to understand the unique IspH-catalyzed transformations, the compositions of the iron−sulfur cluster, and its role in catalysis.

Acknowledgment. This work is supported by the Boston University startup fund for P.L. We thank Professor Dennis Dean's laboratory at Virginia Polytechnic Institute for providing pDB1281 plasmid. We also thank Mr. Lingdong Li for his participation in this work.

Supporting Information Available: Procedures of IspH purification and assays; ^{1}H NMR spectra of **1**, **4**, **5**, **6**; ^{19}F NMR spectra of **4** and fluoride anion (NaF solution); synthetic scheme of **5**. This material is free of charge via the internet at http://pubs.acs.org.

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JA710245D