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Current Development in Isoprenoid Precursor Biosynthesis and Regulation

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Abstract

Isoprenoids are one of the largest classes of natural products and all of them are constructed from two precursors, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). For decades, the mevalonic acid (MVA) pathway was proposed to be the only IPP and DMAPP biosynthetic pathway. This review summarizes the newly discovered IPP and DMAPP production pathways since late 1990s, their distribution among different kingdoms, and their roles in secondary metabolite production. These new IPP and DMAPP production pathways include the methylerythritol phosphate (MEP) pathway, a modified MVA pathway, and the 5-Methylthioadenosine shunt pathway. Relative to the studies on the MVA pathway, information on the MEP pathway regulation is limited and the mechanistic details of several of its novel transformations remain to be addressed. Current status on both MEP pathway regulation and mechanistic issues are also presented.

Keywords

isoprenoids; MVA; MEP; methylthioadenosine; regulation; biosynthesis

INTRODUCTION

Isoprenoids (also known as terpenoids or terpenes) are found in all domains of life (bacteria, archaea, and eukaryotes) and many have important biological functions [1]. For example, quinones act as part of the electron transport chain, sterols as eubacterial and eukaryotic cell membrane components, carotenoids as essential biopigments, dolichol in glycoprotein and bacterial cell wall biosynthesis, and linear prenyl diphosphates as protein prenylation units for intra-cellular protein targeting. In addition, isoprenoids are widely utilized in biomedical and commercial applications including pharmaceuticals, flavoring agents, fragrances, and

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nutrition products. In this paper, we review recent progress made in understanding the biosynthesis of the isoprenoid precursors, dimethylallyl diphosphate (DMAPP, **1**) and isopentenyl diphosphate (IPP, **2**).

BIOSYNTHESIS OF IPP AND DMAPP

The mevalonic acid (MVA) pathway

The mevalonic acid (MVA, **6**) pathway of DMAPP (**1**) and IPP (**2**) biosynthesis (see Figure 1A) has been known for over half a century, and its discovery led to the awarding of the Nobel prize in physiology to Lynen and Bloch in 1964, and in chemistry to Cornforth in 1975 [2]. The pathway starts with the acetyl-CoA acetyltransferase (AACT) catalyzed condensation of two acetyl-CoA (**3**) molecules to form **4**. This is followed by the hydroxylmethylglutaryl-CoA synthase (HMGS) catalyzed reaction to produce 3-hydroxy-3-methyl-glutaryl-CoA (**5**, HMG-CoA). The rate limiting step of the carbon flow through the MVA pathway is the reduction of **5** by HMG-CoA reductase (HMGR) with two equivalents of NADPH. The resulting MVA (**6**) is activated by two phosphorylation steps catalyzed by mevalonate kinase (MVK) and phosphomevalonate kinase (PMK). This is followed by an ATP-coupled decarboxylation of **8** catalyzed by mevalonate pyrophosphate decarboxylase (MPD) to yield IPP (**2**) as the product. Two structurally unrelated IPP:DMAPP isomerases (IDI-1 and IDI-2) are then responsible for the interconversion of IPP (**2**) and DMAPP (**1**) [3,4].

Recently, the existence of a modified mevalonate pathway (Figure 1B) was suggested based on the observation that genes encoding enzymes catalyzing the late steps of the MVA pathway are absent in some archaeal genomes. Grochowski *et al*. [5] identified an enzyme (isopentenyl phosphate kinase, IPK) from *Methanocaldococcus jannaschii*, which is capable of phosphorylating **9** to form IPP. It was thus proposed that **7** is decarboxylated to form **9** and then phosphorylated to produce IPP (**2**). However, the hypothesized decarboxylase (for **7** → **9**) has not yet been identified. Hence, we cannot rule out the option of the existence of a classical MVA pathway in *M. jannaschii*, *while* its PMK and MPD have no or very low sequence similarities to the well-studied PMK and MPD enzymes.

The methylerythritol phosphate (MEP) pathway

In the 1990s and early 2000s, efforts by Rohmer, Arigoni, Lichtenthaler, and Seto etc. led to the discovery of a new isoprene biosynthetic pathway in both bacteria and the chloroplasts of green algae and higher plants [6–11]. This pathway begins with the condensation of pyruvate (**10**) and glyceraldehyde phosphate (**11**) to afford 1-deoxy-D-xylulose 5-phosphate (**12**, DXP) in a reaction catalyzed by the thiamin diphosphate-dependent enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS, see Figure 1C) [12]. DXP is reductively isomerized by DXP reducto-isomerase (DXR, also known as IspC) to 2-*C*-methyl-D-erythritol 4-phosphate (**13**, MEP) [12], from which the MEP pathway receives its name. MEP is then activated by IspD to CDP-ME (**14**) prior to phosphorylation by IspE to produce 4-diphosphocytidyl-2-*C*-methyl-D-erythritol-2-phosphate (CDP-MEP, **15**). Subsequent cyclization of CDP-MEP to 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP, **16**) is catalyzed by IspF. The final two steps are catalyzed by two iron-sulfur containing enzymes, IspG and IspH. IspG is

responsible for the ring-opening and reductive dehydration of MEcPP to produce 4-hydroxy-3-methylbutenyl 1-diphosphate (HMBPP, **17**), whereas IspH catalyzes the reductive dehydration of HMBPP to yield both IPP (**2**) and DMAPP (**1**) (Figure 2C). Thus, in many organisms that utilize the MEP pathway, IDI is not essential for survival, though it may play a role in modulating the IPP/DMAPP ratio in the cell.

Two new activities were recently identified for IspF. Besides the physiological reaction (**15** →**16**), *E. coli* and *Plasmodium falciparum* IspF can also catalyze the conversion of CDP-ME (**14**) to 2-*C*-methyl-D-erythritol 3,4-cyclophosphate (**24**) (Figure 2E) [13,14]. Similarly, the recombinant malaria IspF is capable of catalyzing the production of 2-phospho-2-*C*-methyl-D-erythritol 3,4-cyclophosphate (**25**) and MEcPP (**16**) at a ratio of 1:10 from CDP-MEP (**15**) (Figure 2E) [14]. However, the physiological relevance of these activities remains to be established.

Discovery of a MEP shunt pathway

5-Methylthioadenosine (MTA, **19**) is a side-product of *S*-adenosylmethionine (SAM, **18**)-dependent polyamine biosynthesis, and wild-type *Rhodospirillum rubrum* can grow using MTA as its sole source of sulfur [15]. It is therefore possible that an alternative MTA metabolic pathway is present *R. rubrum* and serves to compensate for the known absence of most canonical methionine salvage pathway genes in this organism. Recently, a ribulose-1,5-bisphosphate carboxylase like protein (RLP), Rru_A1998, was identified by Tabita and co-workers to be an enzyme involved in 5-methylthioadenosine (MTA, **19**) metabolism in *Rhodospirillum rubrum* [16]. Later, Gerlt and co-workders demonstrated that MTA metabolism in *R. rubrum* is coupled to the MEP pathway by supplying DXP (**12**) [15]. In this shunt pathway, MTA is transformed to methylthio-D-ribulose-1-phosphate (MTRu-1P, **21**) by early steps of a canonical methionine salvage pathway that *R. rubrum* is capable of (Figure 2D). MTRu-1P is then converted by Rru_A1998 to a 1:3 mixture of 1-methylthio-ribulose-5-phosphate (MTRu-5P, **22**) and 1-methylthio-xylulose-5-phosphate (MTXu-5P, **23**). A cupin family protein (Rru_A2000) is proposed to catalyze the subsequent C-S lysis reaction in the conversion of MTXu-5P to DXP, producing methanethiol as a co-product, which can be used as the sulfur source (Figure 2D). While the activity of Rru_A2000 has not yet been successfully reconstituted *in vitro*, phylogenetic analysis suggests that a number of organisms could make use of this metabolic shunt to produce IPP and DMAPP [15].

Distributions of MVA and MEP pathways among different kingdoms

The MEP pathway has been identified in eubacteria, green algae, and higher plants, whereas the MVA pathway is found in animals, plants (cytosol), fungi, and archaea [6]. Since the MEP pathway is absent in humans, enzymes involved in this pathway represent excellent targets for development of new broad-spectrum antibiotics and herbicides. This subject has been extensively discussed in many recent reviews [11,17–21].

Although bacteria are known to use the MEP pathway for the production of isoprenoids, there exist exceptions. Several actinobacteria possess genes for both the MEP and MVA pathways [22]. Indeed, feeding experiments using ^{13}C-enriched precursors with

Actinoplanes sp. A40644, Seto and co-workers demonstrated that menaquinones, which are primary metabolites, were derived from the MEP pathway, while the secondary metabolite, BE-406441, was assembled through the MVA pathway [23]. It was suggested that the MEP pathway operates at the early stage of fermentation when the production of primary metabolites is high, whereas the MVA pathway is switched on at a later stage when the production of secondary metabolites is more pronounced. This is consistent with the genes responsible for the MVA pathway being frequently localized to operons for secondary metabolite biosynthesis [24]. Such a genetic organization implies that additional MVA-derived isoprenoid biosynthetic gene clusters may be discovered in bacteria by screening for the highly conserved HMGR gene [24].

Interestingly, a recent study of the biosynthesis of furanonaphthoquinone (**27**, Figure 2), which is a polyketide-terpene hybrid, led to the identification of a mevalonate kinase gene in the furanonaphthoquinone biosynthetic operon, though all the remaining MVA pathway genes were absent [25]. Feeding studies using [^{13}C]-labeled precursors, however, clearly indicated that the terpene portion of **27** (colored red) is derived from the MVA pathway and the quinone portion (**26**, **27**, colored blue) is constructed from **3** by a polyketide synthase [25]. Therefore, the remaining genes of the MVA pathway must be expressed from other regions of the genome. Importantly, the MEP pathway can also serve as the source of the furanonaphthoquinone terpene fragment depending on the culture conditions. As the microbial genome sequencing information continues to expand, additional examples of overlap between the canonical MVA or MEP pathways are expected.

MEP PATHWAY RELATED SIGNALING AND REGULATION

HMG-CoA reductase is the key regulatory step for IPP biosynthesis through the MVA pathway and has been extensively studied [26]. However, studies on the regulation of the MEP pathway and how this pathway interacts with other cellular processes are still at the rudimentary stage and some of the key discoveries are briefly summarized here.

DXR

Analysis of DXR from *Francisella tularensis* suggests that Ser177 (equivalent to Ser185 of *E. coli* DXR) is phosphorylated [27]. In view of the close proximity of Ser177 to the substrate binding site, phosphorylation of Ser177 could be related to DXR activity regulation.

IspF

There exists a central cavity in the crystal structure of IspF, which has been proposed to be the binding site for prenyl phosphate. This may permit regulation of IspF activity through a feedback mechanism [28–30].

IspG

Post-transcriptional regulation of other cellular processes might be achieved through IspG by engaging its iron-sulfur cluster and its substrate, MEcPP (**16**). The [4Fe-4S] cluster in IspG is labile and oxygen sensitive. Under some conditions (e.g., oxidative stress), IspG

may become inactivated leading to the accumulation of MEcPP which may function as an anti-stressor [31]. Interestingly, study of histone-DNA interactions showed that MEcPP can modulate chromosome structures in *Chlamydia trachomatis* [32]. *C. trachomatis* is an intracellular pathogen with a biphasic developmental cycle composed of an infectious extracellular form called the elementary body (EB) and an intracellular replicative form known as the reticulate body (RB). The metabolically inert EB form has condensed nucleoid structure. But within a few hours after infection, the metabolically active RB form will develop and it has the dispersed chromatin. It was suggested that MEcPP can disrupt the histone-DNA interactions and is responsible for the transformation of *C. trachomatis* from the EB form to the RB form. In another report, MEcPP was found to reactivate the "nonculturable" form of *Mycobacterium smegmatis* by triggering the release of HupB, a histone-like protein, from nucleoids to regenerate a transcriptionally active dispersed state [33]. Clearly, IspG plays a pivotal role on the interplay of the MEP pathway with many other physiological processes.

IspH

IspH has been suggested to be part of a global regulation mechanism triggered by elevated levels of guanosine 3',5'-bispyrophosphate ((p)ppGpp) [34,35]. RelA protein is the enzyme involved in the biosynthesis of (p)ppGpp. Gustafson *et al*. proposed that IspH and RelA form a protein complex and the formation of such a complex restricts RelA's (p)ppGpp synthase activity [34]. Under certain conditions, disruption of the RelA-IspH complex would lead to release of RelA protein and increased production of (p)ppGpp. As a result, the elevated level of (p)ppGpp, a nutritional stress alarmone, would trigger a series of energy-consuming metabolic processes, including peptidoglycan synthesis and hydrolysis. However, the details of such an IspH-involved stringent response mechanism remain to be verified.

MECHANICTIC STUDIES ON IspG AND IspH

The MEP pathway exhibits a number of unusual transformations catalyzed by mechanistically unique enzymes. Studies on the mechanisms of these enzymes have become a major focus of research. In this section, we will describe the mechanistic models proposed for IspG and IspH and the most recent biochemical studies on these two enzymes.

2-C-methyl-D-erythritol-2,4-cyclodiphosphate reductase (IspG)

IspG is a [4Fe-4S] cluster-containing enzyme. It is a homodimer and the active site is located at the subunit interface of the two monomers [36,37]. Although progress had been made to improve the *in vitro* activity of IspG using methyl viologen as the reductant [38], the identity of the in vivo reducing system still needs to be established. All current IspG mechanistic models include the assumption that the IspG iron-sulfur cluster contributes to substrate activation and serves as an electron donor for substrate reduction [39]. However, a detailed understanding of the interactions between MEcPP (**16**) and the iron-sulfur cluster remains elusive. This is because the proposed binding site for MEcPP is separated by approximately 20 Å from the iron-sulfur cluster on the other IspG subunit, with which it is proposed to interact, and this would seem to preclude a direct interaction [36,37].

Rohdich *et al*. [40] proposed one of the first IspG mechanistic models, which involves the epoxide 2,3-epoxy-4-hydroxy-3-methyl-butenyl 1-diphosphate (**28**) as an intermediate. In this mechanism, the [4Fe-4S] cluster mediates the reductive deoxygenation of the epoxide **28** to HMBPP (**17**) via the radical intermediate **29** (Figure 3A). This mechanism is supported by the observation that IspG can indeed catalyze the conversion of **28** to HMBPP [41]. However, the rate of **28** → **17** conversion is only 10% of that of **16** →**17** conversion. Thus, **28** may not be a true intermediate and the reductive dehydration of MEcPP and the reductive deoxygenation of **28** may be achieved through parallel pathways [42].

Seemann and Kollas *et al*. instead suggested that IspG-catalysis might involve a carbocation intermediate (**31**, Figure 3B) [43,44]. The observation of a positional isotope exchange for the MEcPP C_2 bridging oxygen provided evidence for a reversible cleavage at this position, which is consistent with the formation of a carbocation intermediate **31** [45]. Based on the characterization of paramagnetic species detected during IspG steady-state turnover, Oldfield and co-workers went on to suggest that IspG catalysis might also involve an organometallic intermediate (e.g., **34**) downstream of the carbocation intermediate **31** [46]. A slightly different version has also been suggested by Duin, Hoffman and co-workders [47]. In these organometallic models, an Fe-C bond forms between the substrate and apical iron of the [4Fe-4S] cluster to yield the intermediate **34.** Because the kinetic competence of this paramagnetic species has not yet been demonstrated, a mechanism involving the radical cation **33** should also be considered as a viable alternative.

4-Hydroxy-3-methyl-butenyl 1-diphosphate reductase (IspH)

IspH is also an iron-sulfur cluster containing enzyme [48] and its iron-sulfur cluster contributes to both substrate activation and serves as an electron donor for substrate reduction [49]. One of two leading IspH mechanistic models is the Birch reduction model [40]. In this model, the reaction is initiated by the coordination of the HMBPP C_4-OH to the $[4Fe\text{-}4S]^{2+}$ apical iron site to yield an HMBPP-IspH complex (Figure 4). Reduction of HMBPP (**17**) by the reduced $[4Fe\text{-}4S]^{+}$ cluster produces the radical anion intermediate **35** (see Figure 4A), which eliminates hydroxide at C_4 to form an allyl radical-$[4Fe\text{-}4S]^{2+}$ intermediate (**36**). Subsequent one-electron reduction may proceed via one of two different routes involving formation of **38** (route I) or **39** (route II) as intermediates. As revealed by a 1.7-Å resolution structure of the IspH-HMBPP complex, HMBPP indeed coordinates to the apical iron site of the [4Fe-4S] cluster using its C_4-OH group [49]. Furthermore, studies using a substrate analog indicate that the HMBPP C_4-OH plays the dominant role in positioning the substrate in IspH active site [50]. The HMBPP olefinic carbons are also positioned adjacent to apical iron site at a distance of 2.8–3.0 Å (Figure 4C, left panel). Thus, the proposed one electron transfer from the iron-sulfur cluster to HMBPP should be feasible.

Recently, a different binding conformation was observed in the structure of the HMBPP-IspH/E126Q mutant. In this case the HMBPP C_4-OH is not coordinated to the [4Fe-4S] cluster, but instead rotates to the other side of the HMBPP double bond to form an internal H-bond with the β-phosphate group (see **40**) [51]. ENDOR spectroscopic analysis of this complex also implicates a similar structure [52]. These observations led to a second

mechanistic model, the organometallic model (Figure 4B and 4C, right panel). Following formation of the initial enzyme-substrate complex, this model predicts the olefin moiety of the substrate to interact with the [4Fe-4S] cluster to form a π complex or the η^2-alkenyl/metallacycle intermediate **40**. In doing so, the free HMBPP C_4-OH rotates to the other side of the molecule to form a H-bond with the β-phosphate group (**40**) and facilitate the subsequent dehydration to **41** (Figure 4B). Results from recent feeding studies using isotopically labeled precursors indicate that such a rotation of the HMBPP C_4-OH is very likely during the IspH catalysis [53].

The Birch reduction and organometallic models are similar in many aspects and share several common intermediates (species **37**, **38**). However, the proposed rotation of the HMBPP C_4-OH and formation of a Fe-C bond involving the olefinic moiety and the [4Fe-4S] cluster are the major points of departure between these two models. In the Birch model, reductive dehydration proceeds in a stepwise one-electron/one-electron transfer manner involving a substrate-based radical intermediate (**36**). By contrast, the reductive C-O bond cleavage step (**40** → **41**) is a two-electron transfer process with concomitant oxidation of $[4Fe\text{-}4S]^+$ to $[4Fe\text{-}4S]^{3+}$ in the organometallic model. Clearly, trapping and characterization of kinetically competent intermediates are essential to distinguish whether IspH catalysis involves stepwise one-electron (the Birch reduction model) or one-step two-electron chemistries (the organometallic model).

SUMMARY

The review provides a brief account of the recent advances in our understanding of the biosynthesis of isoprenoid precursors. While the MVA pathway has been studied for decades, research on the MEP pathway is still at an early stage due to its relatively more recent discovery in the late 1990s. Many questions regarding the interplay between the MVA and MEP pathways, the regulation and control of these pathways, the intracellular distribution of the biosynthetic enzymes involved, and the catalytic mechanisms of the enzymes in the MEP pathway, remain unanswered. These questions are fascinating, albeit challenging, and are being actively pursued by many research laboratories around the world. It is expected that many new and interesting discoveries will result from these efforts with important implications for pharmaceutical and biomedical research.

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- Isoprenoids are widely utilized in biomedical and commercial applications
- Recent progress in understanding the biosynthesis of DMAPP and IPP is reviewed
- While the MVA pathway has been studied for decades, research on the MEP pathway is at an early stage
- Many questions regarding MEP pathways remain unanswered

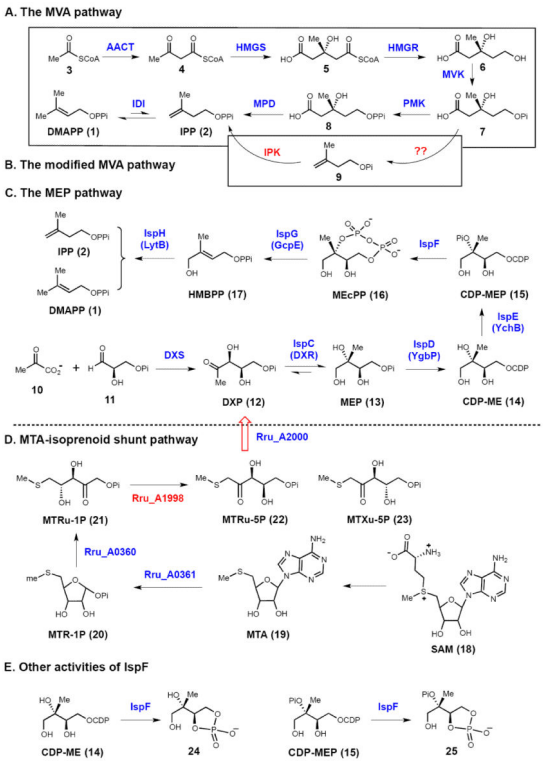

Figure 1.

Pathways for the production of isoprenoid precursors: (A) The mevalonic acid (MVA) pathway. (B) A modified MVA pathway in *Methanocaldococcus jannaschii*. (C) The methylerythritol phosphate (MEP) pathway. (D) A newly discovered isoprenoid shunt pathway related to *S*-adenosylmethioneine metabolism. Two new activities found for IspF are shown in E.

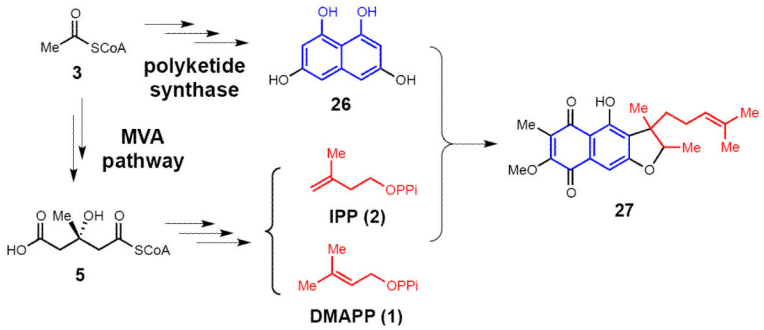

Figure 2.

The involvement of the MVA pathway in the biosynthesis of furanonaphthoquinone (a secondary isoprenoid metabolite) in *Streptoyces cinnamonensis* DSM 1042.

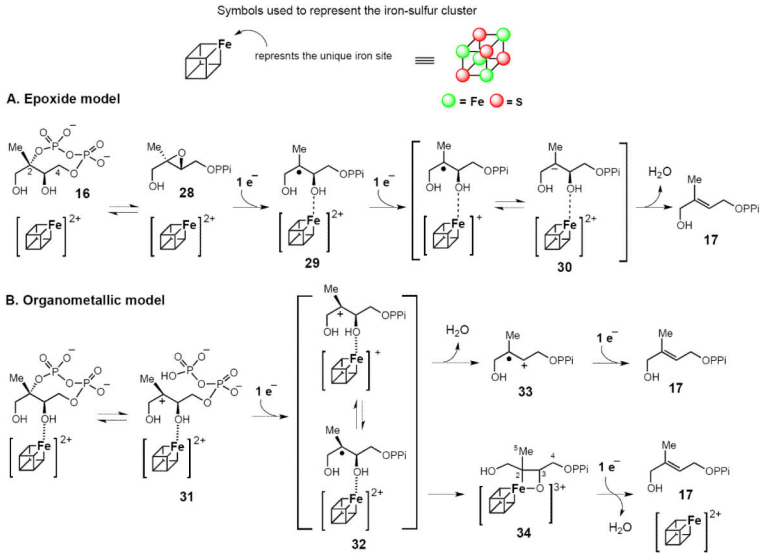

Figure 3.

Working models accounting for the IspG-catalyzed reaction. (A) The epoxide model. According to this model, the epoxide (**28)** serves as a key intermediate. Once **28** is formed, two sequential single electron reduction steps lead to the formation of HMBPP (**17**). (B) Cation and organometallic models. In this case, the first step is the formation of a carbocation intermediate (**31**) by the C-O cleavage. Subsequent reductive dehydroxylation can proceed via either the radical cation intermediate (**33**) or the organometallic intermediate (**34**).

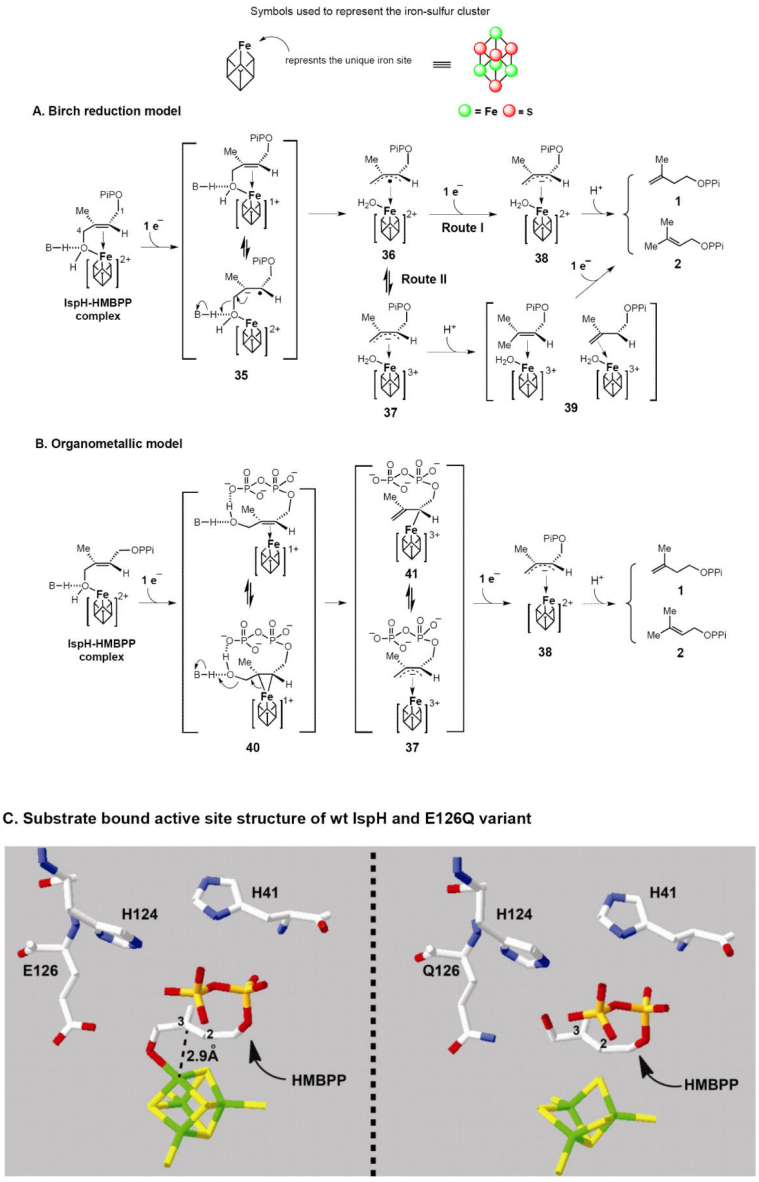

Figure 4.

Working models accounting for the IspH-catalyzed reaction. (A) Birch reduction model. In this model, the iron-sulfur cluster has two functions: (1) mediating two sequential one-electron reduction, and (2) serving as the Lewis acid to facilitate C_4-dehydroxylation. (B) Organometallic model. This model has two unique features: (1) the HMBPP C_4-OH group rotates away from the [4Fe-4S] cluster to form an internal H-bond (**40**), and (2) an iron-sulfur cluster mediated two-electron reductive dehydroxylation step (**40** → **41**). (C) Substrate bound active site structure of wt and E126Q variant IspH.