Isoprenoid Biosynthesis

DOI: 10.1002/anie.201000833

Reductive Dehydroxylation of Allyl Alcohols by IspH Protein

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deoxyxylulose phosphate pathway · iron–sulfur clusters · isoprenoid biosynthesis · IspH protein · LytB protein

The biosynthesis of natural products is a treasure trove of unusual reaction mechanisms. This Minireview summarizes recent work on the structure and mechanism of IspH protein, which catalyzes the reductive dehydroxylation of an allyl alcohol in a biosynthetic pathway leading to isoprenoid precursors.

1. Introduction

Isoprenoids are the largest group of known natural products (more than 35000[1]), including numerous medically important compounds such as cholesterol, steroid hormones, vitamins, and drugs. They are all assembled from two isomeric five-carbon compounds, isopentenyl diphosphate (IPP, **7**) and dimethylallyl diphosphate (DMAPP, **8**). These two precursors can be generated either through the mevalonate pathway or the more recently discovered non-mevalonate pathway.

The amount of biomatter obtained from IPP and DMAPP is daunting. For example, about 500 megatons of isoprene (C_5H_8), the smallest biosynthetic isoprenoid, are secreted by coniferous trees per year and are a major contributor to the formation of atmospheric aerosols.[2]

The mevalonate pathway, a key chapter in virtually every biochemistry textbook, was elucidated by pioneering studies in the 1950s.[3] Three acetate moieties, in the form of acetyl-CoA (**11**), afford one molecule each of CO_2 and IPP. Enzyme-catalyzed isomerization of the latter species yields DMAPP.

The hydroxyglutaryl-CoA reductase of the mevalonate pathway is the target of the statin drug family designed for the prevention and treatment of cardiovascular disease by lowering the endogenous biosynthesis of cholesterol.[4] Global sales exceeded 26 billion US$ in 2008,[5] by far the biggest market share of any single drug family.

The impressive achievements made in mevalonate research eclipsed the existence of a second isoprenoid pathway for several decades. However, following the pioneering discoveries by Rohmer, Arigoni, and co-workers in the 1990s,[6] the intermediates, the enzymes, and the cognate genes of the alternative, non-mevaloante pathway (Scheme 1) were discovered in rapid succession around the turn of the century.[7–10] The emergent discipline of comparative genomics played a central role in that development.[11]

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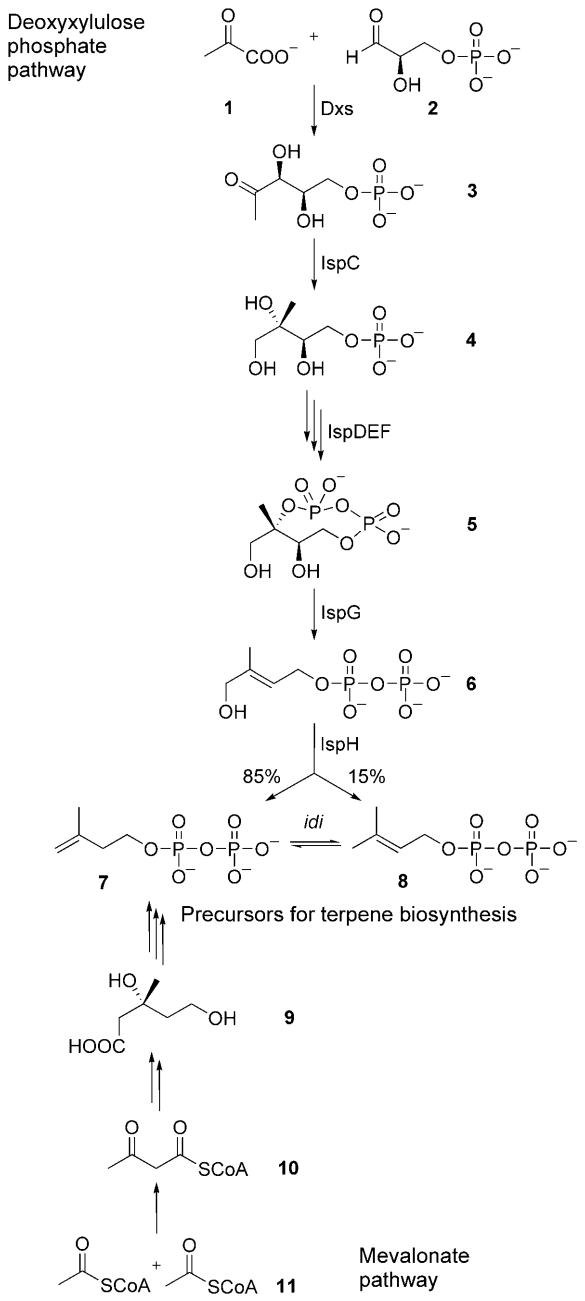
2. The Terminal Step of the Non-Mevalonate Pathway

In the mevalonate pathway the condensation of acetoacetyl-CoA (**10**) with acetyl-CoA (**11**) directly leads to formation of a branched C_5 skeleton. In the non-mevalonate pathway, the C_5 skeleton is initially generated in a linear form, and branching is subsequently introduced by a skeletal isomerization. More specifically, the condensation of glyceraldehyde phosphate (**2**) with pyruvate (**1**) affords 1-deoxyxylulose phosphate (**3**),[8,12] which is converted into 2*C*-methyl-D-erythritol 4-phosphate (**4**) by a sequence of isomerization and reduction catalyzed by IspC (Dxr) protein.[9] The polyol phosphate is then converted into 2*C*-methyl-D-erythritol-2,4-cyclodiphosphate (**5**) via cytidine diphosphate intermediates by a sequence of three enzyme-catalyzed steps (Scheme 1).[7,13]

As a consequence of its origin from a carbohydrate precursor, the cyclic diphosphate intermediate **5** has an excess of oxygen substituents compared to IPP and DMAPP. In the absence of suitable activating substituents, the removal of the excess oxygen substituents is a mechanistic hurdle that is overcome by enzyme-mediated radical reactions. Specifically, the eight-membered ring of **5** is opened reductively by IspG protein, which has a [4Fe-4S] cluster.[14] The product, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate (HMBPP, **6**), is then converted by the iron–sulfur protein IspH into a 6:1 mixture of IPP and DMAPP.[15–18] This product ratio differs



Scheme 1. Isoprenoid biosynthesis.[20]

significantly from the thermodynamic equilibrium, which is characterized by a ratio of 1:3. The product of the kinetically controlled IspH reaction can be brought to thermodynamic equilibrium by the action of IPP isomerases;[19] the IspH protein itself, however, has no isomerase activity.

The transformation of **6** into IPP and DMAPP requires two electrons. In eubacteria, flavodoxin reductase and flavodoxin serve as an electron transponder chain for IspG and/or IspH protein.[18,21] In fact, flavodoxin is an essential protein in *Escherichia coli*, and the flavodoxin requirement can be suppressed by the implementation of an engineered cluster of mevalonate biosynthesis genes.[21] The physiological electron transponder(s) for the iron–sulfur proteins of the non-mevalonate pathway in blue-green algae and in plant chloroplasts are not yet completely known; ferredoxin has been reported to form complexes with and provide electrons to IspG protein of *Thermosynechocystis elongatus* and to IspH protein of *Plasmodium falciparum*.[22]

The handling, purification, and crystallization of IspH protein is hampered by the inherent oxygen sensitivity of its iron–sulfur cluster.[16,17] All operations must therefore be performed under strictly anaerobic conditions. The history of the reported specific activity values is testimony to the experimental difficulties (Figure 1). The highest catalytic

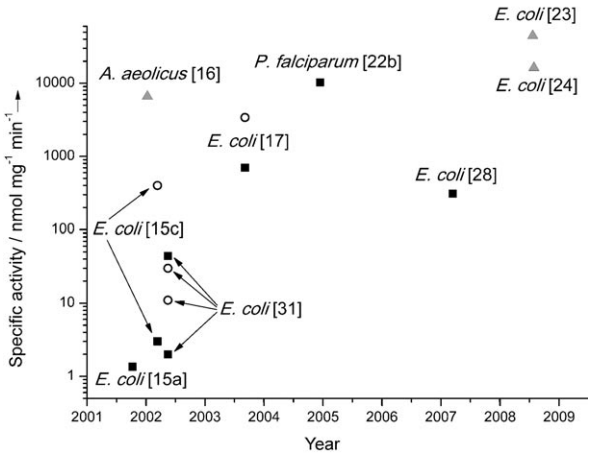
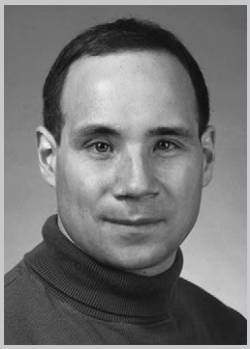


Figure 1. History of specific activities reported for IspH protein using NADPH (■), methylviologen (▲), or photoactivated deazaflavin (○) as electron transponders. References are shown in brackets.



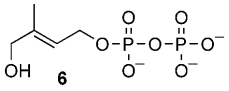
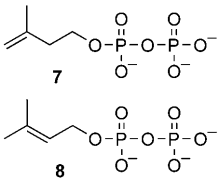
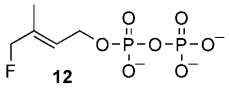
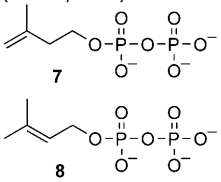
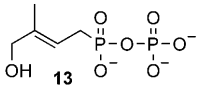
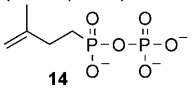
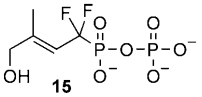
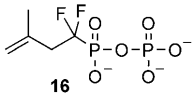
Michael Groll was born in 1971 in Donauwörth, Germany. After receiving his PhD for crystallographic and biochemical studies on the 20S proteasome, he joined Prof. Huber for postdoctoral studies. In 2002 he joined the group of Prof. Finley at Harvard Medical School. After running an independant group at the Ludwig-Maximilians-Universität München, he became professor for Biochemistry at the Humboldt-University of Berlin. Since 2007 he has held the Chair of Biochemistry at the TU München. His research focuses on the functional and structural characterization of multifunctional protein complexes.

Tobias Gräwert was born in Munich in 1977. From 1998 he studied biology at the TU Munich, where he also received his diploma in 2003 and his PhD in 2006 under the supervision of Prof. Bacher. In 2009, he began his Habilitation in the laboratory of Prof. Groll. His research focuses on the characterization of iron–sulfur proteins as well as on the search for inhibitors of the non-mevalonate pathway.

rates were reported with artificial electron transponders such as methylviologen.[16,23,24] In the absence of an electron donor, the stability of the protein is considerably enhanced by an excess of substrate.[25]

Stereochemical studies and use of substrate analogues have provided some important clues on the reaction mechanism of IspH. In D_2O as solvent, deuterium is stereospecifically incorporated into the H_{Si} position at C3 of IPP.[26] The 1-fluorine-substituted substrate analogue **12** is converted by IspH protein into a mixture of IPP and DMAPP, with a product ratio (7:1) resembling that observed with the natural substrate, HMBPP (6:1; Table 1).[27] Structural analogues with certain modifications at C4 (**13**, **15**) can serve as substrates, but afford only one product isomer (**14** and **16**, respectively) in detectable amounts.[27,28]

Table 1: Reactions catalyzed by IspH protein.

Substrate	Product	k_{cat} [min^{-1}]	K_M [μM]	Ref.
 **6**	 **7** + **8** (ratio **7**/**8** 6:1)	11.6	< 15	[27]
 **12**	 **7** + **8** (ratio **7**/**8** 7:1)	0.55	3950	[27]
 **13**	 **14**	0.44	< 15	[27]
 **15**	 **16**	0.022	n.d.	[28]

3. Crystal Structures of IspH

Recently, crystal structures have been obtained for IspH protein of *E. coli*[23,25] and of the hyperthermophilic eubacterium *Aquifex aeolicus* (Figure 2).[29] Strikingly, the *E. coli* enzyme could so far only be crystallized with an anionic ligand bound at the active site, whereas IspH protein of *A. aeolicus* crystallized without a specific ligand bound at the active site.

The IspH proteins of *A. aeolicus* and *E. coli* are both monomeric, and their folding patterns involve three closely similar domains (D1–D3) that are related by pseudo-C_3 symmetry, although these domains have no detectable sequence similarity. The structure is without precedent in the literature. Each folding domain comprises four strands

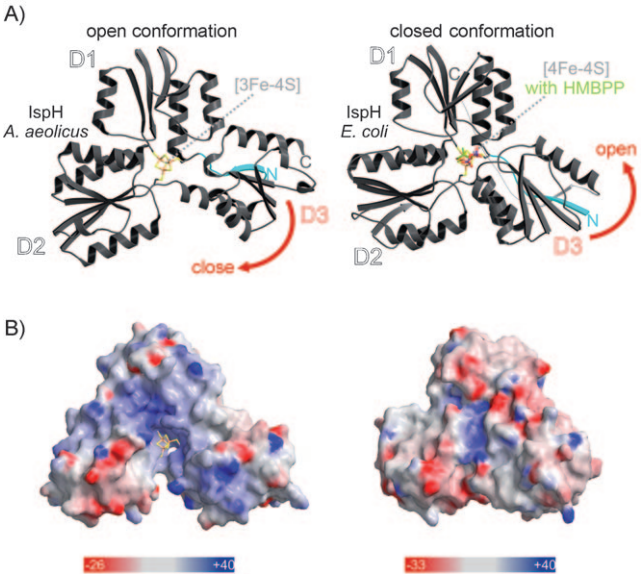


Figure 2. Open and closed conformations of IspH protein. Left: IspH of *A. aeolicus*[29] (without bound ligand in the active site cavity, PDB ID: 3DNF); right: IspH of *E. coli*[25] (PDB ID: 3KE8). A) Ribbon plots; red arrows indicate the putative motion of the domain D3 during transition between the open and closed conformations. B) Space-filling models with electrostatic potentials (in kTe^{-1}).

(S1–S4) arranged in a central parallel β sheet, which is flanked by the α helices H1–H3 (Figure 2).

In the case of the *E. coli* protein, the pseudo-threefold arrangement of the individual domains is stabilized by a characteristic clamp motif formed between the N and the C terminus. The resulting parallel β sheet completes the cyclic domain arrangement, since the stringently conserved N terminus[23,29] contributes the S4 strand in domain D3 and proceeds into helix H1 of D1 (Figure 2A). The deletion of 11 N-terminal or 30 C-terminal amino acids resulted in unsoluble protein, thus indicating the importance of this clamp motif for structural integrity.[23]

Cysteine residues coordinating the iron–sulfur cluster are located at N-terminal positions of each folding domain. They are followed by the respective H1 helices, whose macrodipole vectors converge at the location of the [3Fe-4S] cluster, where they generate a positive Coulomb potential[23] that may stabilize the reduced state of the cluster, as determined by comparison with inorganic model systems. As a consequence of the somewhat different domain topology, the *E. coli* protein features a closed central cavity, whereas the *A. aeolicus* protein presents an open conformation where the central cavity is freely accessible to the bulk solvent.[23,29]

X-ray crystal structures of the *E. coli* protein have now been obtained in a complex with 1) an early intermediate, 2) a late intermediate whose precise chemical structure is still unknown, 3) the two enzyme products IPP and DMAPP, 4) inorganic pyrophosphate, and 5) malonate.[23] The polyanion moieties of bound intermediates occupy the same topological position inside the central cavity, where they are embedded in a polar environment comprising the side chains of histidines 41, 74, and 124, serines 225 and 269, threo-

nine 168, asparagine 227, and glutamine 166. The diphosphate esters are all bound in a hairpin-shaped conformation, with the organic moiety sandwiched between the pyrophosphate moiety and the iron–sulfur cluster.

4. The Iron–Sulfur Cluster

The stoichiometry of the iron–sulfur cluster at the active site is still controversial in IspH research. The structures of the *E. coli* protein comprise [4Fe-4S] clusters complexed with the early or late reaction intermediate. On the other hand, [3Fe-4S] clusters were consistently observed in the complexes of the *E. coli* enzyme with IPP, DMAPP, inorganic pyrophosphate, or malonate.[23,25] Also of note is that the *Aquifex* enzyme crystallizes in the open conformation with a [3Fe-4S] cluster.[29]

Mössbauer and EPR spectroscopy had earlier suggested the presence of [4Fe-4S] clusters (Figure 3) in the catalytically active enzyme.[30–32] Mössbauer spectra of intact *E. coli* cells containing large amounts of recombinant IspH protein have also been interpreted in terms of [4Fe-4S]-IspH.[30] On the other hand, a [3Fe-4S] stoichiometry has been reported for catalytically active *E. coli* on the basis of EPR experiments.[17] An emerging consensus on the controversial iron content could imply the following factors:

1. The [4Fe-4S] cluster enables a reaction trajectory via an alkoxide intermediate (see Scheme 2B).
2. The apical cluster iron ion that is not coordinated by any amino acid can be easily lost from [4Fe-4S]-IspH; in the absence of electron donors, complexation with the substrate significantly reduces the oxygen sensitivity of *E. coli* IspH, possibly by protection of [4Fe-4S] clusters.[25]
3. The isolation of IspH protein from recombinant hyperexpression strains may afford mixtures of [3Fe-4S] and [4Fe-4S] protein if the cellular iron–sulfur cluster assembly machinery has insufficient capacity to cope with massive apoprotein expression; this may even be the case in expression strains that have been engineered for the coexpression of the *isc* operon specifying enzymes for iron–sulfur cluster biosynthesis.[17,33]
4. Similarly, the in vitro reconstitution of active IspH protein from apoprotein may afford mixtures of molecular species with different cluster stoichiometries.[29,31]
5. Crystallization conditions may select protein species with different clusters, depending on the nature of the ligands used for the cocrystallization and on the species of origin.
6. Since at least some catalytic activity has been reproducibly demonstrated with dissolved IspH:pyrophosphate cocrystals, whose [3Fe-4S] status appears to be solidly established by crystallography,[23] it remains possible that the [3Fe-4S] protein can undergo dismutation which results in the regeneration of the [4Fe-4S] cluster; however, the possibility that [3Fe-4S]-IspH, per se, could also display some catalytic activity through a reaction trajectory different from the emerging [4Fe-4S]-IspH reaction mechanism (see Scheme 2) has not been ruled out with full certainty.

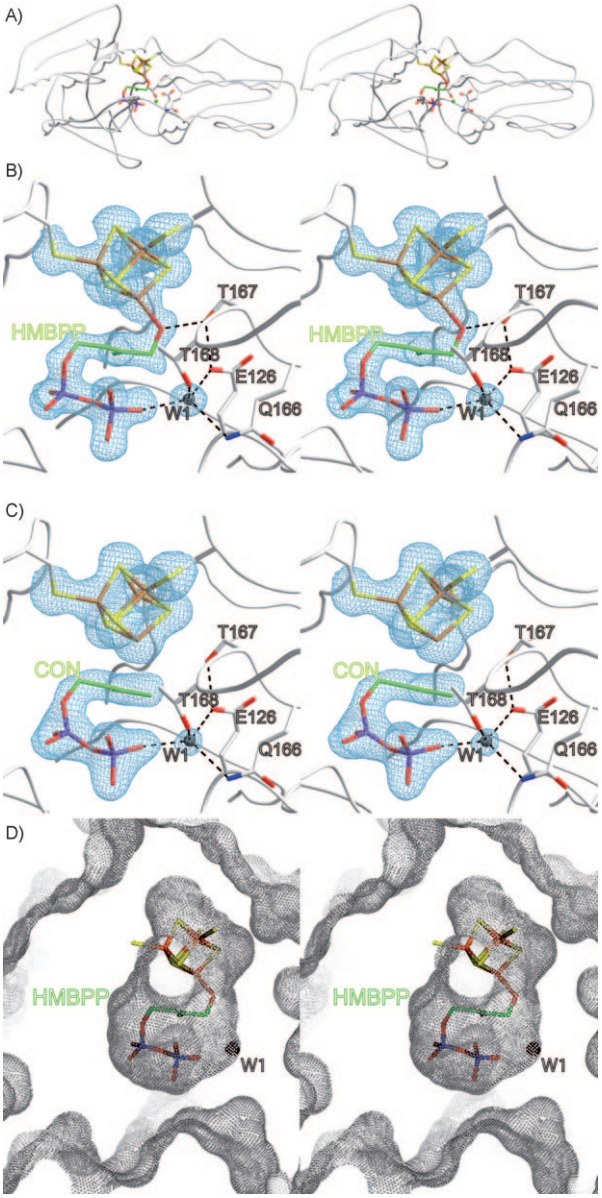
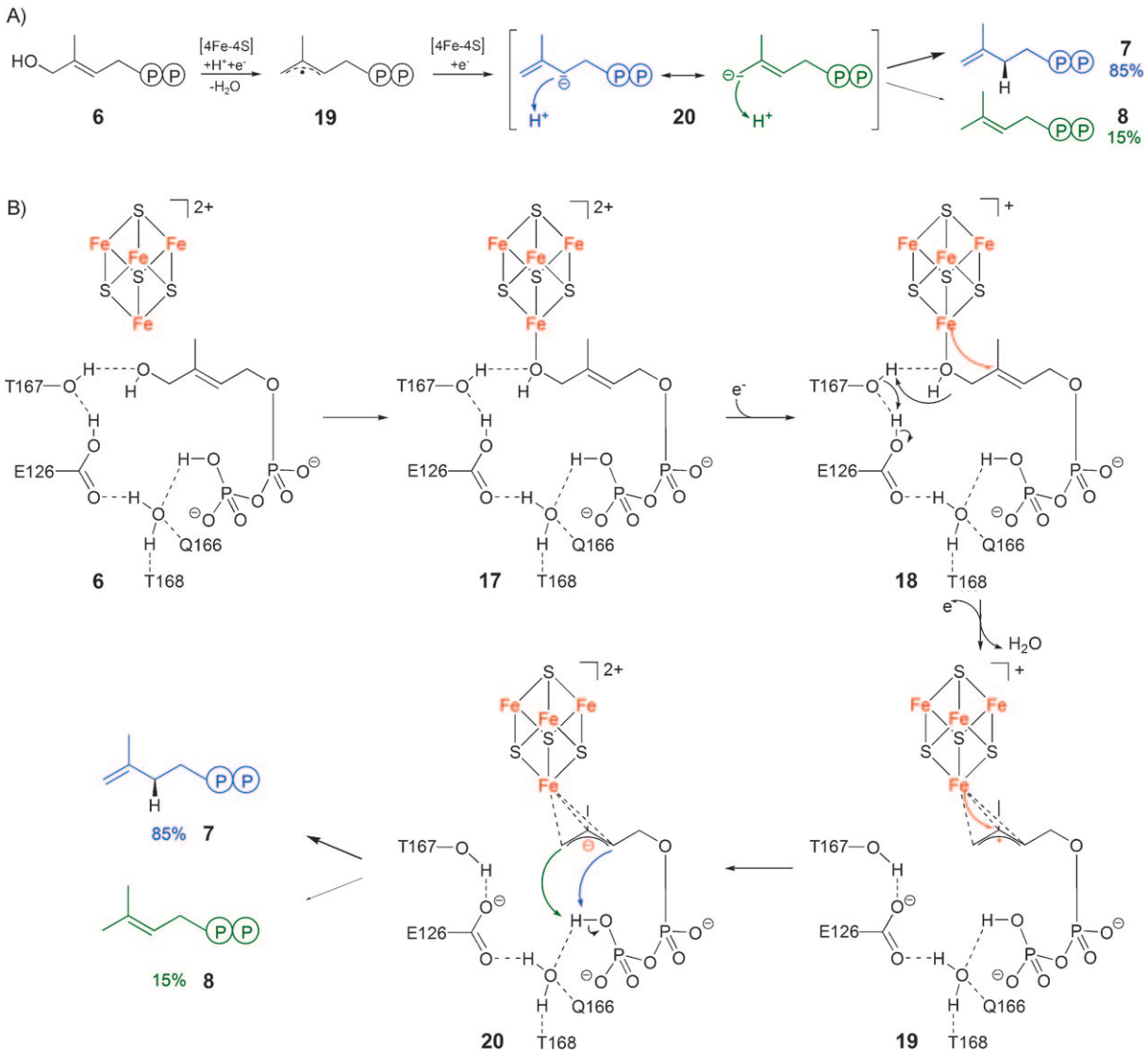


Figure 3. Snapshots from the IspH reaction trajectory (stereopairs).[25] A) Overview; B) "early reaction intermediate" at the active site; electron density (blue) contoured at 1.0 σ with ($2F_o-F_c$) coefficients (PDB ID: 3KE8); C) CON: "late reaction intermediate" (enzymatic transformation initiated by CuK_α preirradiation of a IspH:substrate crystal; PBD ID: 3KE9); D) Connolly map of the IspH central cavity harboring the W1 water molecule, an iron–sulfur cluster, and the early reaction intermediate.

5. Mechanistic Considerations

On basis of these data, a hypothetical reaction mechanism for IspH can be developed in some detail (Scheme 2B). The formation of an alkoxide (**17**) from the hydroxy group at C1 of the substrate and the apical iron ion of a [4Fe-4S] cluster may be the first reaction step (Figure 3B). The iron–oxygen distance (2.0 Å) is well within the range for an iron–oxygen bond. The distances of the apical iron ion from C2 and C3 of the enzyme-bound isoprenoid intermediate **17** (2.8 and 2.9 Å, respectively) are significantly below the van der Waals



Scheme 2. Reaction mechanism of IspH catalysis. A) Experimentally established chemical details. B) Hypothetical model for the interactions of the reactants at the surface of the closed active site cavity; cluster charges according to Wang et al.[36]

distance (around 3.6 Å) and suggest at least some σπ interaction between the iron ion and the bound alkene moiety.

In the absence of a reducing agent, the early reaction intermediate **17** (Figure 3B) could not progress any further along the reaction trajectory. However, when an IspH:HMBPP cocrystal was exposed to CuK_α radiation and then stored at 70 K for an extended period prior to collecting a diffraction data set by using synchrotron radiation, the photoelectrons generated by the CuK_α irradiation were able to drive the reaction further down the reaction trajectory. It is as yet unknown whether the resulting X-ray structure (Figure 3C) reflects the formation of a reaction intermediate downstream from the alkoxide complex **18** or of final product (IPP/DMAPP). The initiation of chemical transformation by X-ray irradiation of enzyme:substrate complex crystals has precedent in studies on DNA photolyase.[34]

Starting from the alkoxide complex **17** in Scheme 2B, the most likely scenario is that the transfer of one electron to the alkene motif and of a proton to the oxygen atom at C1 can prepare the early reaction intermediate **18** for the cleavage of the carbon–oxygen bond at C1 (but it is unknown whether the electron and proton transfer occur simultaneously or sequentially). Clearly, the electron would have to be transferred from or via the iron–sulfur cluster. More specifically, whether the electron can be provided by a change in the oxidation state of the cluster ([4Fe-4S] clusters can accomodate 0, +1, +2, or +3 net charge[35]) or whether an external electron source is strictly required at this stage of the reaction trajectory may depend on the redox state of the cluster at the beginning of the reaction.

The protein shown in Figure 3B was clearly unable to propel the substrate down the reaction trajectory because of the absence of an electron donor. However, photoelectrons generated by X-ray irradiation enabled the reaction to proceed to the cleavage of the carbon–oxygen bond at C1 (Figure 3C, Scheme 2B). However, the absence of a reducing agent (Figure 3B) is an artificially generated situation; inside

wild-type bacterial cells, IspH protein operates in the presence of its natural electron transponder cascade.[18]

The transfer of a proton could occur from the carboxylic group of Gln 126 via a hydrogen bond network that involves Thr 167 and the alkoxide intermediate **18**. The replacement of Glu 126 by Gln has been shown to reduce the activity of IspH protein by at least a factor of 100.[23] The concept of heterolytic cleavage of a carbon–oxygen bond is strongly supported by the observation that the fluorine analogue **12** can serve as a substrate for IspH protein; a homolytic cleavage would be impossible in the case of a carbon–fluorine bond. The similar product ratios observed with the natural substrate **6** and the fluoro analogue **12** leave no doubt that the reaction trajectories for both compounds are essentially the same.

The heterolytic cleavage of the carbon–oxygen bond affords an allyl radical (**19**) which may be stabilized by σπ interactions with the iron–sulfur cluster.[25] Incidentally, the absence of a kinetic isotope effect with [4-$^2H_2$]-**6** and the conversion of the substrate analogues **13** and **15** into IPP analogues provide conclusive evidence against the participation of a dienyl intermediate in the reaction trajectory.[27,28]

There is precedent in the literature for the facilitation of carbon–oxygen bond cleavage by the prior generation of a radical state.[37] The most closely investigated example is nucleotide reductase, where the removal of the hydroxy group at position 2 of a ribofuranose moiety is enabled by the prior abstraction of a hydrogen atom from the adjacent C3-position.

The final stage of the IspH reaction trajectory requires the transfer of a second electron from an external electron source, which results in the formation of an allyl anion (**20**) that may again benefit energetically from stabilization by σπ interactions with the iron–sulfur cluster (Scheme 2 B). The resulting mesomeric allyl anion **20** can be converted into either of the IspH products by the transfer of a proton to C1 (affording DMAPP, **8**) or C3 (affording IPP, **7**). This reaction step appears to involve a substantial decrease in the free energy (not surprising since allyl anions are very strong bases), thus making it practically irreversible. It would otherwise be difficult to explain the kinetic control of the product ratio and the absence of IPP isomerase activity[17]

Stereochemical evidence indicating that a solvent proton is incorporated into the $C3_{Si}$-position provides an important steric constraint for reprotonation.[26] In light of the hairpin conformation of the bound intermediate, the intramolecular pyrophosphate moiety is a plausible proton donor for IPP formation.

6. IspH—An Allosteric Enzyme?

E. coli IspH has virtually the same conformation in all available X-ray structures (rmsd < 0.4 Å, even though structures were determined from crystals with different space groups). These closed conformations fail to explain the entry of the substrate and the export of product, since the active site cavity is completely closed and impermeable (Figures 2 B and 3 D). The open conformation structure of the *A. aeolicus* protein suggests a plausible answer to this dilemma.[29]

The topologies of the D1 and D2 domain ensembles are closely similar in the *E. coli* and *A. aeolicus* structures. On the other hand, the domain D3 is tilted by about 20° in the *Aquifex* protein compared to in the *E. coli* structures (Figure 2 A). A simple, dynamic hinge may involve the backbone elements of the amino acids Phe 11 and Cys 197.[23] Thus, IspH can be compared to a "nutcracker" that closes around the substrate, reductively cleaves off a hydroxy group, and finally opens up to release the product.[23]

The superficially simple transition between open and closed conformations must involve a complex choreography of water molecules. During formation of the early reaction intermediate shown in Figure 3 B and D, the substrate and the surface of the active site cavity must be stripped almost entirely of their hydration water. In the final phase of the catalytic cycle, the active-site surface (Figure 3 D) and the product need to be rehydrated. Whereas the cognate entropic and enthalpic contributions may essentially cancel each other, any residual net contribution may provide an important role for stabilization of the transition state, which deserves further analysis.

The redox state of this enzyme prior to substrate binding is oxidized, thus two electrons are required to complete the reductive reaction in the sealed active-site cavity. Therefore, the active cavity becomes more electronegative. This charge modulation may play a role in the opening of the cavity and the release of the product at the end of the reaction trajectory.

7. Conclusions

In summary, catalysis by IspH protein may benefit from a trias of protein conformational motion, water exclusion, and protonation of the reaction intermediate for stabilization of the transition state of IspH protein. This is reminiscent of findings with the chalcone isomerase of *Medicago sativa*.[38]

The non-mevalonate pathway is the single source of essential isoprenoids in the majority of pathogenic bacteria (with Gram-positive cocci as the major exception) and for the apicoplast subgroup of pathogenic protozoa.[39] Hence, the enzymes in this pathway are considered as potential targets for the therapy and/or prevention of major killer diseases such as tuberculosis and malaria.[40] Understanding the details of the reaction mechanism and the conformational modes of IspH protein should facilitate the discovery of inhibitors with therapeutic potential.

We thank the Hans-Fischer-Gesellschaft for financial support.

Received: February 10, 2010
Published online: October 4, 2010

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