Biosynthesis

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Structures of Fluoro, Amino, and Thiol Inhibitors Bound to the [Fe_4S_4] Protein IspH**

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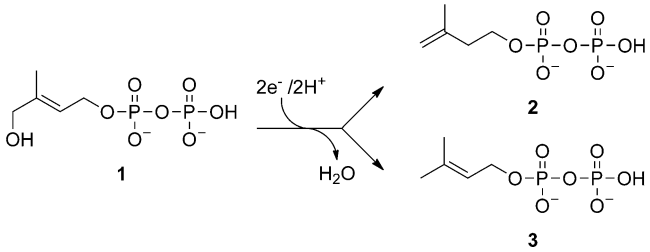
Isoprenoids derive from two universal precursors: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP).[1] In mammals, these key intermediates are crucial for cell survival, for example, in cholesterol biosynthesis, and are generated by the mevalonate pathway. In contrast, in most bacteria as well as in malaria parasites such as *Plasmodium falciparum*, the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway is used.[2] The DXP pathway is absent in humans and is, therefore, considered to be an important drug target against many infectious diseases.[3]

Scheme 1 shows the last step of the DXP pathway, the conversion of (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP, **1**) to a mixture of IPP (**2**) and DMAPP (**3**). This reaction is catalyzed by HMBPP reductase (IspH), a monomeric enzyme that consists of three domains binding to a redox-active [Fe_4S_4] cluster in its central cavity (Figure 1 a).[4] The cluster is linked to the protein by the side chains of three cysteine residues, and one of the iron atoms possesses an unoccupied coordination site that has not been found to bind to any amino acid residue. Mechanistic studies with wild-type *Escherichia coli* IspH as well as IspH mutants



Scheme 1. Reductive dehydration catalyzed by IspH.

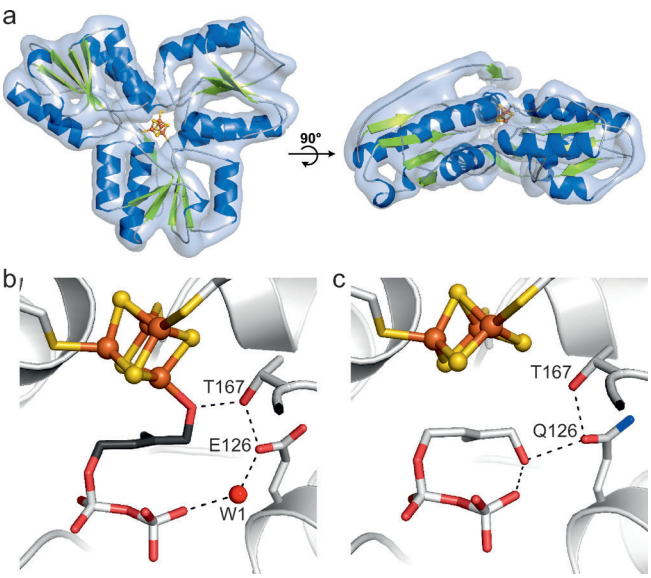


Figure 1. Crystal structure of IspH in complex with **1**. a) Top and side views of the overall structure of *E. coli* IspH. Active site of b) wild-type IspH forming an alkoxide complex with **1** and c) the IspH E126Q mutant bound to **1** in the cyclic conformation revealing a [Fe_3S_4] cluster.

have revealed two different conformations of **1** inside the active site that are adopted in the catalytic cycle (Figure 1 b and c): one in which O1 binds to the fourth iron atom, and a second in which the hydroxy group of **1** undergoes numerous hydrogen-bond interactions with the diphosphate group and protein residues.[5]

The proposed mechanism for the IspH reaction is shown in Scheme 2 and involves four intermediate states that have been identified by crystallography, Mössbauer spectroscopy, and electron paramagnetic resonance (EPR) spectroscopy.[5,6] The detailed structure of IspH in the absence of exogenous

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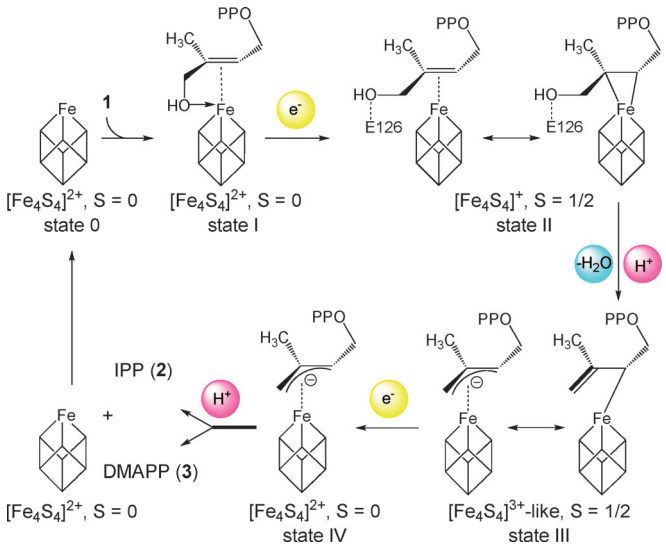
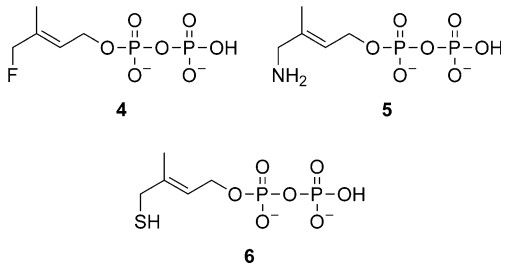


Scheme 2. Proposed mechanism of IspH catalysis.

ligands is not known (state 0) but the binding of **1** to oxidized IspH leads to the formation of an alkoxide complex with weak π interactions (state I; spin $S=0$). One-electron reduction of the cluster results in $[Fe_4S_4]^+$ with spin $S=1/2$ and correlates with a rotation of the ligand's hydroxymethyl group away from the cluster to form a cyclic conformation (state II), which has important implications for the stereochemical course of the IspH reaction.[7] The transfer of two electrons from the cofactor to the substrate produces a HiPIP-type $[Fe_4S_4]^{3+}$ cluster and leads to C–O bond cleavage and water release. The allyl anion (state III) then abstracts a proton from the diphosphate group, at either the ligand's C2 or C4 atom, to form IPP and DMAPP, respectively.

In addition to the intensive investigation of the IspH reaction mechanism, tremendous effort has been invested in the design and characterization of inhibitors.[8] Recently, the synthesis and spectroscopic studies of three substrate analogues have been reported in which the hydroxy group in HMBPP is replaced by fluoro (**4**),[9] amino (**5**),[10] or thiol (**6**) groups (Scheme 3). Compound **4** is slowly converted by IspH,



Scheme 3. Analogues of the IspH substrate **1**.

whereas **5** and **6** inhibit the enzyme. In order to analyze the structure–function relationship of these derivatives we synthesized **4**,[11] **5**,[12] and **6** (see the Supporting Information), performed co-crystallization with *E. coli* IspH, and determined the crystal structure of the complexes.

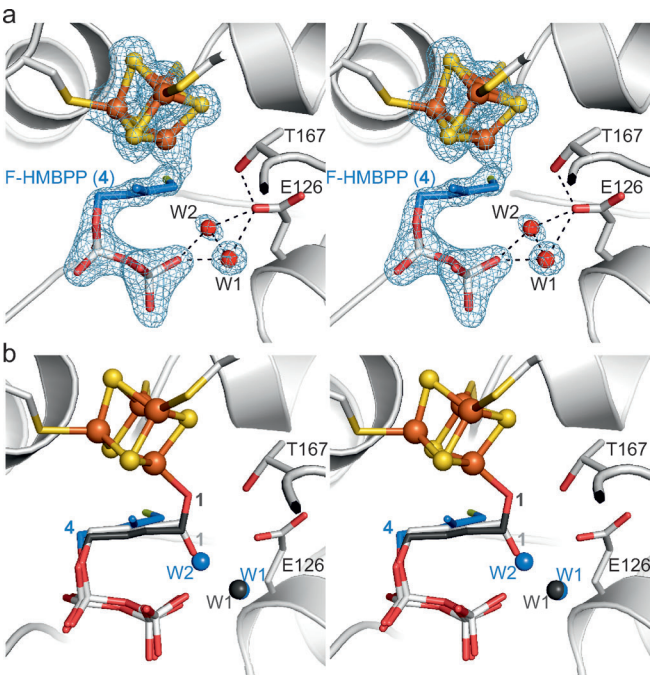


Figure 2. Complex structure of IspH bound to the fluorinated derivative **4**. a) Active site of IspH showing the bound ligand and two water molecules. A $2F_O-F_C$ omit electron density map (blue mesh, contoured at 1.0 σ) is shown for the $[Fe_4S_4]$ cluster, the ligand, and the solvent molecules in the first coordination sphere; dotted lines indicate hydrogen bonds. b) Structural superposition of IspH:**4** with the alkoxide complex (IspH:**1**) and the cyclic intermediate (IspH E126Q:**1**).

The X-ray structure of IspH in complex with the fluoro analogue **4** was determined to 1.8 Å resolution [$R_{free}=23.2\,\%$, Figure 2a, Protein Data Bank (PDB)[13] ID 4H4C] and reveals that **4** binds to the active site of IspH in a similar way as the substrate **1**.[14] However, the C–F bond is rotated by 106° compared to the C–O bond in the IspH:**1** complex (Figure 2b); the fluorine atom is thus located inside a hydrophobic pocket and is stabilized by van der Waals interactions with $His74C^{\delta}$ (3.6 Å), Ala73C (3.9 Å), and $Ala73C^{\beta}$ (3.9 Å). This unique conformation allows water molecules to occupy positions W1 and W2.[14] Although it displays an unusual orientation, **4** is converted to **2** or **3** by IspH, but at a lower rate ($k_{cat}=28\ min^{-1}$) than **1** ($k_{cat}=604\ min^{-1}$). The differences in these reaction rates are likely due, at least in part, to the increased bond energies of C–F versus C–O.[15] Furthermore, the lack of a direct interaction with the apical iron atom leads to the high K_m value of **4** ($K_m=104$ μM) compared to that of **1** ($K_m=20$ μM).

Recent inhibition studies have shown that the amino and thiol substrate analogues **5** and **6** exhibit potent inhibition of IspH with IC_{50} values of 0.15 μM and 0.21 μM, respectively.[10] Additionally, Mössbauer spectroscopy has suggested that both ligands interact with the $[Fe_4S_4]$ cluster. However, it is not immediately obvious that **5** binds to the fourth iron atom through its amino group, or whether it forms an alternative complex that allows a water molecule to coordinate to the fourth iron atom, as previously observed with an acetylene inhibitor.[8c]

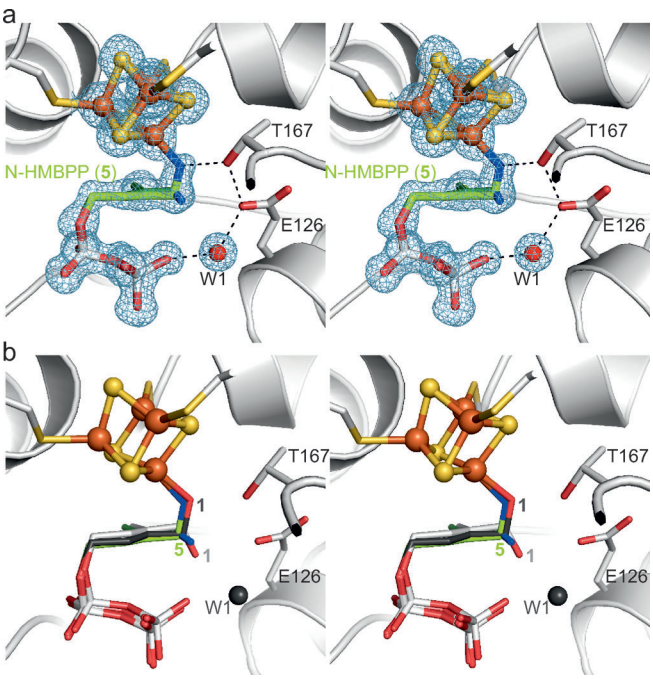
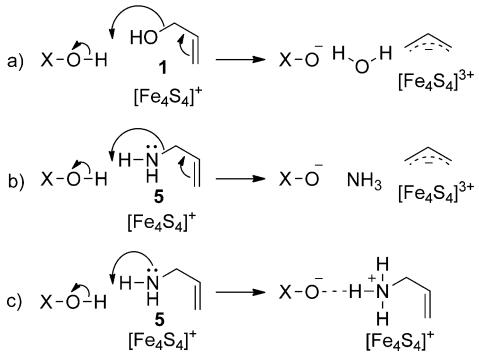


Figure 3. X-ray structure of IspH in complex with the amino derivative **5**. a) Active site with the ligand in two orientations. The electron density map is displayed in analogy to Figure 2a. b) Superposition of the IspH:**5** complex structures with the alkoxide complex in IspH:**1** and the cyclic intermediate in IspH E126Q:**1**.



Scheme 4. Mechanism of IspH catalysis with **1** and inhibition by **5**. a) Dehydroxylation of **1**, facilitated by an acidic proton donor (Glu126OH or the diphosphate) represented by X-OH. b) Corresponding hypothetical reaction with **5**. c) Formation of a stable ammonium–carboxylate ion pair.

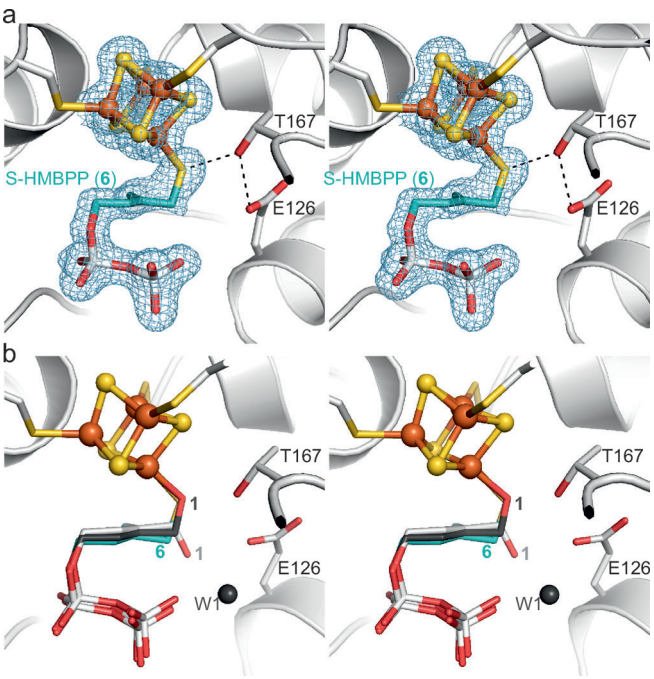


Figure 4. IspH bound to the thiol derivative **6**. a) Active site with the thiol–iron complex. The electron density map is displayed in analogy to Figure 2a. b) Structural superposition of IspH:**6** with the alkoxide complex in the IspH:**1** structure and the cyclic ligand orientation in the IspH E126Q:**1** complex.

The structure of **5** in complex with IspH was determined to 1.35 Å resolution (R_{free} = 21.0%, Figure 3a, PDB[13] ID 4H4D) and clearly shows two ligand conformations within the same crystal:[16] 1) a ligand–cluster complex in which the amino group coordinates to the apical iron atom and 2) a conformation in which the amino group is rotated by approximately 74° in the opposite direction to that observed with **4**. The amino–iron complex is similar to that seen with the alkoxide–iron complex formed by **1** (Figure 3b), indicating that the affinity of the free amino group to the $[Fe_4S_4]^{2+}$ cluster is comparable to that of the hydroxy group. The second conformation observed in the crystal structure is stabilized by hydrogen bonding of the amino (or ammonium) group to one of the diphosphate oxygen atoms (2.8 Å), Glu126O$^{\varepsilon}$ (2.9 Å), and Thr167O$^{\gamma}$ (3.1 Å), and to a water molecule in the W1 position (3.1 Å).

The amino–iron complex is in good agreement with the Mössbauer spectroscopic data indicating a tetrahedral (3S/N) coordination sphere at the apical iron atom.[10] Thus, it seems likely that the alternative conformation may have arisen by cluster reduction in the X-ray beam—a result that would be of particular interest in the context of the inhibition of the IspH reaction which is, of course, carried out under reducing conditions.[17] However, unlike with **1**, no turnover was observed with **5**. One possible explanation for this is the formation of a stable ammonium–carboxylate ion pair that prevents the release of ammonia (Scheme 4).

Next, we determined the crystal structure of IspH bound to **6** to 1.7 Å resolution (R_{free} = 21.3%, Figure 4a, PDB[13] ID 4H4E), which displays complex formation between the unique iron atom of the cluster and the thiol group of **6**. No solvent molecule is located in the active site, since the increased size of the bridging group leads to movement of the hydrocarbon chain of **6** towards the diphosphate moiety. Furthermore, the hydrogen-bond network is modified, preventing the stabilization of the water molecules in the same positions as those found in the IspH:**1** complex (Figure 4b). The coordination by the ligand's thiol group generates an $[Fe_4S_4]$ cluster that is coordinated by four sulfur ligands, similar to that found in most proteins catalyzing electron-transfer reactions.[18]

In conclusion, the results presented here provide new insight into the mechanism of the IspH reaction with the artificial substrate **4**, which involves a previously unknown intermediate that is not bound to the $[Fe_4S_4]$ cluster. With the amino (**5**) and thiol (**6**) species, we find in both cases that the ligands bind to the fourth iron atom. However, it still remains to be determined whether IspH inhibition is due to bonding of these ligands to the oxidized protein, to the reduced protein, or to both. With **5**, we see evidence for a second conformation in which the side chain does not interact with the cluster and propose the formation of a nonreactive ion-pair complex with $Glu126O^{\varepsilon}$ and the diphosphate group. As expected, in the IspH:**6** complex, the ligand's sulfur atom binds to the fourth iron atom, and there is no alternative conformation, most likely because the SH group is a poor base/hydrogen-bond acceptor. The structures of these substrate analogues thus provide new mechanistic insights and also suggest novel strategies for the development of antibiotic and antimalarial drugs.

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[14] The crystal structure of IspH bound to **4** shows excess (approximately 10%) electron density between the fluorine atom and the apical iron atom due to partial hydrolysis to **1** during crystallization, with **1** coordinating to the iron–sulfur cluster.
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[16] X-ray crystallography is an averaging method, which enables the determination of atomic coordinates of an ensemble of molecules. The IspH:**5** complex structure displays two ligand conformations within the same crystal, which means that one part of the ensemble adopts one conformation, whereas the other part of the ensemble displays another conformation.
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