Organometallobiochemistry

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Isoprenoid Biosynthesis: Ferraoxetane or Allyl Anion Mechanism for IspH Catalysis?**

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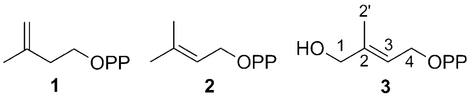
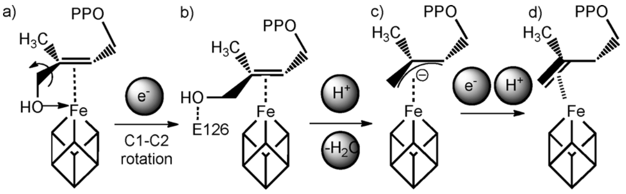
There are around 65000 terpenes known.[1] These molecules are produced from two isoprenoid (C_5) diphosphates: isopentenyl diphosphate (IPP, **1**) and dimethylallyl diphosphate (DMAPP, **2**).[2] In plant plastids, IPP and DMAPP are produced primarily through the 2-*C*-methylerythritol 4-phosphate (MEP) pathway from *E*-1-hydroxy-2-methyl-but-2-enyl 4-diphosphate (HMBPP, **3**) in a reaction catalyzed by the enzyme IspH, and this route is also used in most bacteria, as well as in malaria parasites (Scheme 1).



Scheme 1. IspH products and substrate.

There is thus interest in understanding the mechanism of action of IspH since this could lead to new inhibitors of interest as anti-infective drug leads. IspH is a 4Fe–4S cluster-containing protein and catalyzes the deoxygenation of HMBPP through a $2H^+/2e^-$ reduction.[3] There has been considerable debate over the IspH mechanism of action with cationic, anionic, radical, butadiene as well as organometallic hypotheses being proposed.[4] Most of these proposals have now been ruled out, but there is considerable evidence in support of the organometallic hypothesis.[5] In this hypothesis, it is proposed that HMBPP initially binds to the oxidized ($[Fe_4S_4]^{2+}$) cluster through O1 (in the CH_2OH group; Scheme 2a). On cluster reduction, the CH_2OH group rotates about C1−C2 and moves away from the cluster to interact with the highly conserved E126 (*E. coli* residue numbering), and a diphosphate OH group, which can provide the H^+ required for dehydroxylation, and subsequent ligand protonation. The intermediate that forms is stabilized by a π interaction between the alkene group and the cluster (Scheme 2b). An internal $2e^-$ transfer then ensues, resulting in a "super-oxidized" or HiPIP (high-potential iron–sulfur protein, $[Fe_4S_4]^{3+}$)-like $S = {}^1\!/_2$ cluster and an allyl anion (Scheme 2c) which after addition of a second electron and H^+ yields the IPP/DMAPP products and the original oxidized cluster (Scheme 2d).



Scheme 2. Proposed allyl anion mechanism for IspH catalysis.

More recently, a second organometallic mechanism has been proposed[6] for IspH in which another species, a ferraoxetane, is involved. This intermediate is analogous to the ferraoxetane intermediate we previously proposed for IspG catalysis[7,8] and would contain Fe−O as well as Fe−C bonds. In addition, these workers have proposed another alternative, oxa-allyl, model for IspG catalysis.[9] In essence, then, there are two sets of models: the allyl anion model for IspH[4,5] together with the ferraoxetane model for IspG[7,8] that we have proposed (Scheme 3a,b), and the ferraoxetane model for IspH together with the oxa-allyl model for IspG[9] (Scheme 3c,d). What is not clear from the recently proposed IspH ferraoxetane mechanism[6] (Scheme 3c) is how DMAPP could form, and how this mechanism can be reconciled with the observation that the CH_2OH group rotates away from the cluster, as deduced from both crystallographic and isotope-labeling experiments.[10,11] We have thus investigated the chemical nature of the newly reported IspH reaction intermediate[6] in more detail by using HYSCORE[12] spectroscopy with ^2H, ^{13}C and ^{17}O-labeled substrates and one-electron

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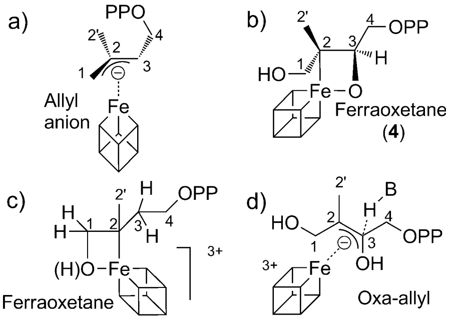

Scheme 3. Proposed structures of reaction intermediates involved in IspH and IspG catalysis. The structures we have proposed are a) for IspH and b) for IspG;[4,5,7,8] the structures from alternative proposals are c) for IspH and d) for IspG.[6,9]

reduced *Aquifex aeolicus* IspH, which yields an intense EPR spectrum characterized by $g_{11} = 2.17$.[6]

With IspG, we previously reported the results of HYSCORE and ENDOR[13] experiments using nine ^{2}H, ^{13}C and ^{17}O-labeled substrates that produced the intermediate "X", proposed to be the ferraoxetane **4** (Scheme 3b). There were three key observations that led to this structural proposal: First, the HYSCORE spectrum of "X" prepared using an ^{17}O-labeled substrate exhibited a large (ca. 8 MHz) isotropic hyperfine interaction. This coupling is very similar to those seen with $H_2{}^{17}O$ bound to the unique, 4th Fe in the 4Fe–4S cluster in aconitase (8.65 MHz), and most hyperfine couplings for systems containing Fe–O bonds are in the range 8–15 MHz.[14–16] Second, there was a ca. 17 MHz hyperfine coupling observed for the quaternary carbon (C2), while all other carbons had much smaller hyperfine couplings (< 4 MHz), indicating formation of a Fe–C2 bond.[7,8] Third, the large (ca. 12 MHz) ^{1}H hyperfine coupling observed in the absence of any isotopic labeling was shown to arise from a single hydrogen in the C2′ methyl group[8] since, using a CD_3-labeled substrate, we found three signals, one ($A_{iso}(^2H) = 1.7$ MHz) corresponding to the $A_{iso}(^1H) \approx 12$ MHz signal. These isotope-labeling experiments (and the results of DFT calculations) suggested that "X" was the ferraoxetane **4**.[7]

The structure of **4** (Scheme 3b) is clearly similar to that now proposed for the $g_{11} = 2.17$ IspH reaction intermediate[6] (Scheme 3c), the exception being of course that there are differences in the positions of the H/alkyl groups (since the substrates are different). It seems implausible that these would have major effects on electronic structure, in which case two predictions for the IspH ferraoxetane model would be: 1) a large 1-^{17}O hyperfine coupling and 2) a large 2-^{13}C hyperfine coupling. With the allyl anion model we proposed previously,[4,5] the predictions would be: 1) no hyperfine interaction with ^{17}O (since it is not present), and 2) there should be three ^{13}C hyperfine couplings, one to each of the three carbons in the allyl anion.

We first obtained EPR and HYSCORE spectra of reconstituted, one-electron reduced *Aquifex aeolicus* IspH (AaIspH) with [1-^{17}O]-HMBPP (that is, labeled at the 1, CH_2OH position), since in the recently proposed IspH ferraoxetane model this group is directly bonded to the unique 4th Fe in the 4Fe–4S cluster.[6] The EPR spectrum was essentially the same as that we reported previously for a sample prepared from *E. coli* IspH in the presence of excess dithionite and HMBPP, having $g_{11} = 2.17$, $g_{22} = 2.01$, and $g_{33} = 1.99$ (Figure 1a). The HYSCORE spectrum is shown in Figure 1b and there is no evidence for any ^{17}O signal. These new results, therefore, do not provide support for the IspH ferraoxetane hypothesis but are consistent with formation of an allyl anion complex (in which the ^{17}O has been lost).

Next, we used a uniformly ^{13}C-labeled HMBPP to produce the $g_{11} = 2.17$ species. There was no evidence for a large (ca. 17 MHz) ^{13}C hyperfine coupling (Figure 1c and Figure S1 in the Supporting Information). There were, however, three sets of small hyperfine couplings (denoted C(A), C(B), and C(C) in Figure 1c). To assign these resonances, we synthesized [1-^{13}C]-, [2-^{13}C]- and [3-^{13}C]-HMBPPs, and obtained HYSCORE spectra of the $g_{11} = 2.17$ intermediates. The hyperfine couplings could be simulated using EasySpin[17] with $A_{ii}(A) = [2.0,\ 0.5,\ 6.7]$ MHz; $A_{ii}(B) = [-1.1,\ -0.8,\ 7.3]$ MHz, and $A_{ii}(C) = [7.3,\ 0.9,\ 0.9]$ MHz (Figures S2 and S3), corresponding to $A_{iso} = 3.1$, 1.8, and 3.0 MHz, respectively. As can be seen in Figure 1d–f, the HYSCORE spectrum of the [1-^{13}C]-HMBPP labeled sample corresponds to the C(C) ($A_{iso} \approx 3.0$ MHz) signal, the [2-^{13}C] sample corresponds to C(A) ($A_{iso} \approx 3.1$ MHz), and the spectrum of the [3-^{13}C]-HMBPP labeled sample corresponds to C(B) ($A_{iso} \approx 1.8$ MHz).

Next, we investigated the HYSCORE spectra of two ^{2}H-labeled HMBPPs: [2′-$^2H_3$]-HMBPP and [4-$^2H_2$]-HMBPP, bound to IspH (as the $g_{11} = 2.17$ reaction intermediate). In previous work[5] we obtained HYSCORE spectra of (±)-[1-$^2H_1$]- and [3-^{2}H]-HMBPP complexed with reduced EcIspH (the same $g_{11} = 2.17$ reaction intermediate) under turnover conditions. Both exhibited clear ^{2}H hyperfine couplings with $A_{iso} \approx 0.9$ MHz and 0.5 MHz, respectively, for the (±)-[1-$^2H_1$]- and [3-^{2}H]-HMBPP ligands (Figure S4a, b) The HYSCORE spectra of one-electron reduced *A. aeolicus* IspH samples (again, characterized by identical g_{ii} values as samples prepared by freeze-quenching) with [2′-$^2H_3$]-HMBPP and [4-$^2H_2$]-HMBPP are shown in Figure S4c, d. In both cases, ^{2}H hyperfine couplings are present, but they are clearly very small, inconsistent with the expectation that one deuteron in the methyl group in a ferraoxetane would have a large hyperfine coupling, as seen in IspG. The hyperfine couplings seen in IspG[7,8] are shown on the ferraoxetane structure in Figure 2a; the new experimental results for IspH are shown on the IspH ferraoxetane model in Figure 2b, and the experimental results for IspH are shown again on the allyl anion model, in Figure 2c. In the IspG ferraoxetane (Figure 2a), both atoms bonded to Fe (O3, C2) have large hyperfine couplings. Similar couplings would be expected for an IspH ferroxetane but there is actually no ^{17}O signal observed, and the C2 hyperfine coupling tensor is $A_{ii} = [2.0,\ 0.5,\ 6.7]$ MHz, to be compared with $A_{ii} = [14.5,\ 12.0,\ 26.5]$ MHz in IspG.[8,9] These results support the allyl anion π-complex model (Figure 2c) in which there is no ^{17}O hyperfine coupling because the ^{17}O has already been removed, as water, and there are three small ^{13}C hyperfine couplings (to C1, C2 and C3 in an allyl anion). These ^{17}O and

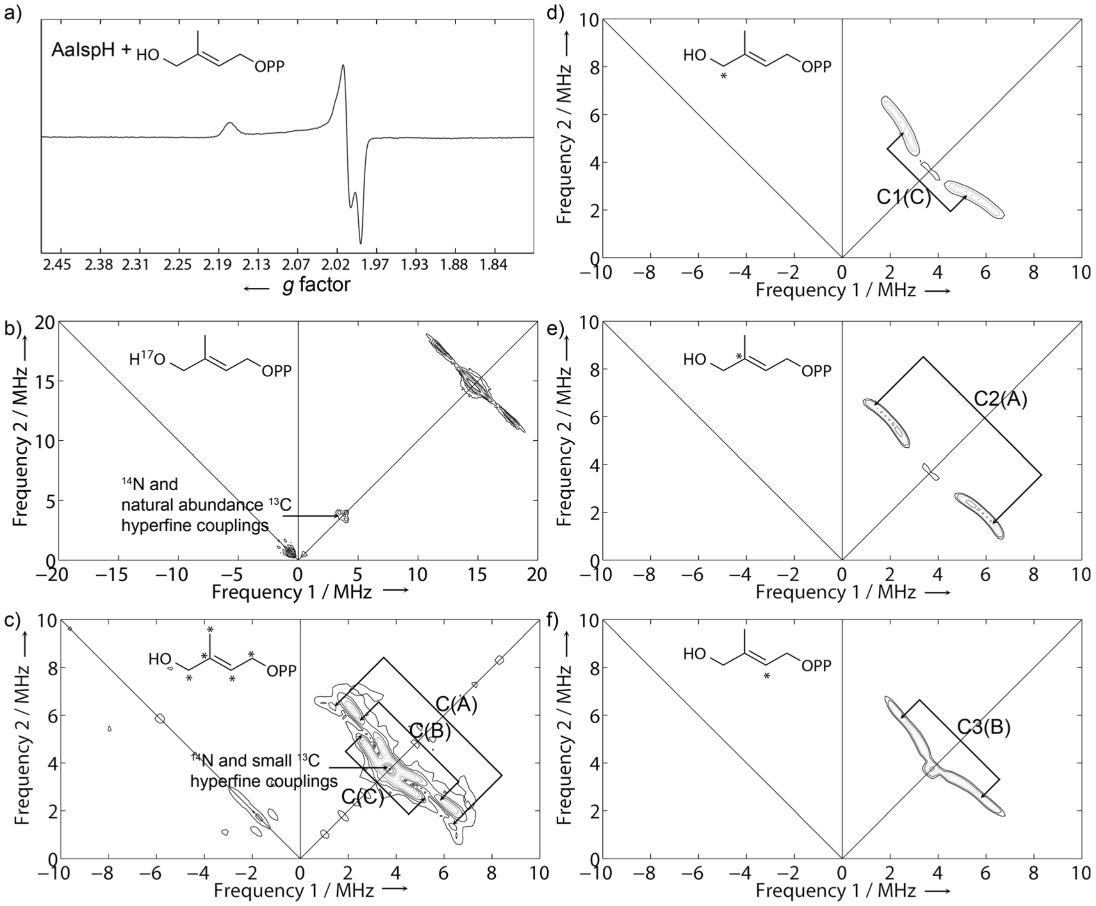

Figure 1. a) 9.05 GHz continuous-wave EPR spectrum of one-electron reduced *A. aeolicus* IspH + HMBPP. b) 9.68 GHz HYSCORE spectrum of one-electron reduced AaIspH + [1-^{17}O]-HMBPP, sum of spectra with $\tau = 128$, 182 and 236 ns, $B_0 = 344.4$ mT. c) 9.68 GHz HYSCORE spectrum of one-electron reduced AaIspH + [*U*-$^{13}C_5$]-HMBPP, sum spectra with $\tau = 128$, 150, 166, 182, 196, and 208 ns, $B_0 = 344.7$ mT. d) 9.69 GHz HYSCORE spectrum of one-electron reduced AaIspH + [1-^{13}C]-HMBPP. e) 9.69 GHz HYSCORE spectrum of one-electron reduced AaIspH + [2-^{13}C]-HMBPP. f) 9.69 GHz HYSCORE spectrum of one-electron reduced AaIspH + [3-^{13}C]-HMBPP. d–f) are all sum spectra with $\tau = 136$, 160, and 196 ns, $B_0 = 344.1$ mT. All spectra were taken at 10 K.

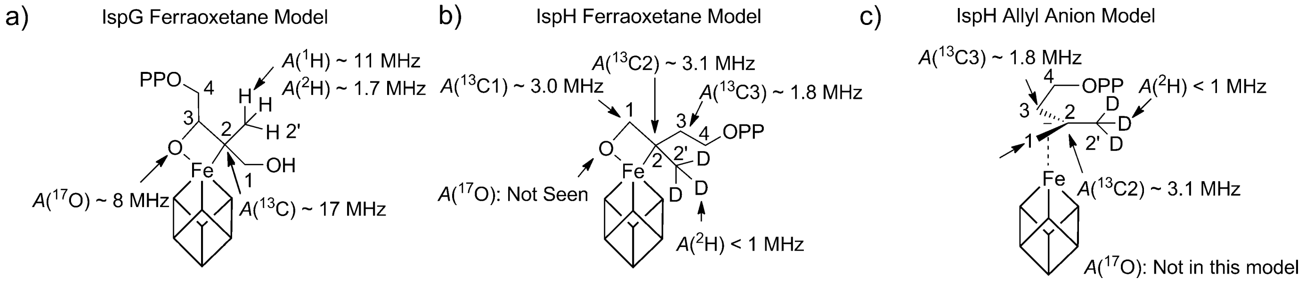

Figure 2. Hyperfine couplings (A_{iso}, MHz) observed for reaction intermediates in IspH and IspG catalysis based on HYSCORE and/or ENDOR spectra with isotopically labeled substrates.

^{13}C results are also supported by current and previous ^{2}H HYSCORE/ENDOR spectroscopic results. With the IspG ferraoxetane (Figure 2a), there is one very large ^{2}H/^{1}H hyperfine coupling, due to a single proton in the CD_3/CH_3 group. There is no corresponding feature in the IspH reaction intermediate that would be expected from the structure shown in Figure 2b, and the couplings that are seen are larger for H1, H3 (in the allyl group) than they are for H2′ and H4. The lack of any large ^{2}H, ^{13}C or ^{17}O hyperfine couplings, together with the three observed ^{13}C HYSCORE signals, are all supportive of an allyl anion IspH intermediate[5] and not a ferraoxetane.[6]

The question then arises as to whether the $g_{11} = 2.17$ species corresponds to the intermediate observed crystallographically.[18,19] In the latter the average distance from Fe to C1, C2 and C3 is 2.8 ± 0.3 Å. Using the hyperfine tensor results and the point dipole approximation with the spin projection coefficients for aconitase,[20] we obtained a range of

$2.0(\pm0.2)$ to $2.5(\pm0.2)$ Å. To try to obtain more accurate results, we next investigated the allyl anion/Fe-S cluster structure using DFT. We used a $[Fe_4S_4(SMe)_3(\eta^3\text{-}CH_2\text{-}C\text{-}(CH_3)\text{-}CH(CH_2OH)]$ cluster with $S = 1/2$, with methods reported previously,[21–23] using the Gaussian09 program.[24] After full geometry optimization (Figure 3a), the distances between the unique 4th Fe and C1, C2, and C3 were 2.14, 2.10, and 2.17 Å, respectively. The computed A_{iso} values were in quite good accord with experiment: $A_{iso}(C1) = 3.0$ MHz (expt.), 1.91 MHz (calc.); $A_{iso}(C2) = 3.1$ MHz (expt.), 2.87 MHz (calc.); A_{iso} (C3) = 1.8 MHz (expt.), 1.70 MHz (calc.) (Table S1 and Figure S5). These results all indicate that the $g_{11} = 2.17$ species is the $[Fe_4S_4]^{3+}$-like/allyl anion species III[5] (Figure 3b, left) while the species observed crystallographically is likely a weakly bound product that forms through IV (Figure 3b, middle), based on the ca. 2.8 Å bond lengths seen in the X-ray structure (Figure 3b, right).

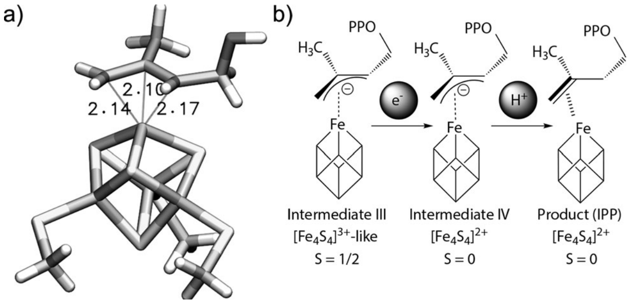

Figure 3. IspH intermediate structures and mechanism. a) The model used to compute hyperfine tensors. b) Reduction and protonation of the $[Fe_4S_4]^{3+}$-allyl anion results in product formation with a proposed weak π-interaction between the double bond in the IPP product and the 4th Fe.

In summary: the results reported here are of interest for several reasons. First, using a [1-^{17}O] HMBPP substrate we find no evidence for Fe−O bonding in an IspH reaction intermediate prepared by using a one-electron reduced cluster, making a ferraoxetane intermediate unlikely. Second, using a uniformly ^{13}C-labeled HMBPP substrate, we find no evidence for the large (ca. 17 MHz) hyperfine interaction seen previously with IspG and attributed there to a ferraoxetane intermediate. Rather, we detected three signals, all with quite small hyperfine couplings. Third, using [1-^{13}C], [2-^{13}C] and [3-^{13}C]-labeled HMBPP substrates, we were able to specifically assign all three signals. Fourth, the ^{13}C hyperfine couplings observed were in good accord with DFT predictions. Fifth, we find no evidence for the large hyperfine interaction seen previously with the IspG (ferraoxetane) reaction intermediate due to a single proton in the C2′ CH_3 group. Overall, the results are of broad general interest since they help clarify the nature of the $g_{11} = 2.17$ reaction intermediate observed in IspH catalysis, highlighting the unusual, organometallic mechanism of the reaction. Moreover, the results are consistent with both crystallographic[10] and isotope-labeling[11] experiments which indicate C1−C2 bond rotation in the allyl anion model that would not occur with the ferraoxetane mechanism, plus, the allyl anion mechanism provides a route to DMAPP formation that is absent in the ferraoxetane mechanism.

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