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Bioorganometallic Chemistry with IspG and IspH: Structure, Function and Inhibition of the [Fe₄S₄] Proteins Involved in Isoprenoid Biosynthesis

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Abstract

The methylerythritol phosphate pathway of isoprenoid biosynthesis is an attractive anti-infective drug target. The last two enzymes of this pathway, IspG and IspH, are [Fe₄S₄] proteins not produced by humans that catalyze 2H⁺/2e[−] reductions with novel mechanisms. In this review, we summarize recent advances in structural, mechanistic and inhibitory studies of these two enzymes. In particular, mechanistic proposals involving bioorganometallic intermediates are presented and compared with other mechanistic possibilities, and inhibitors based on substrate analogs, developed by rational design and compound library screening, are discussed. These results represent the first examples of bioorganometallic catalytic mechanisms of [Fe₄S₄] enzymes, and open up new routes to inhibitor design targeting [Fe₄S₄] clusters.

Keywords

Bioinorganic chemistry; Biophysics; Enzyme catalysis; EPR spectroscopy; Terpenoids

1. Introduction

Iron-sulfur proteins containing [Fe₄S₄] clusters^[1] carry out a remarkably diverse series of reactions ranging from electron transfer (in ferredoxins)^[2, 3] to dehydration/isomerization (in e.g. aconitase in the Krebs's cycle)^[4, 5] to free radical chemistry performed by enzymes belong to the radical-SAM (*S*-adenosyl methionine) superfamily^[6]. More recently, another class of [Fe₄S₄] proteins was discovered that catalyze formation of two key intermediates in isoprenoid biosynthesis: isopentenyl diphosphate (IPP, **1**; Scheme 1) and dimethylallyl diphosphate (DMAPP, **2**). The reactions involved are the 2H⁺/2e[−] reductions of 2-*C*-methyl-D-erythritol-2,4-*cyclo*-diphosphate (MEcPP, **3**) to *E*-1-hydroxy-2-methyl-but-2-enyl-4-diphosphate (HMBPP, **4**), catalyzed by IspG (also known as GcpE), and the 2H⁺/2e[−] reduction of **4** to form **1** and **2**, catalyzed by IspH (also known as LytB). IspG and IspH are both present in the methylerythritol phosphate (MEP) isoprenoid biosynthesis pathway (also known as the non-mevalonate pathway) discovered some 20 years ago,^[7] and are essential for the survival of most bacteria as well as of malaria parasites, *Plasmodium* spp., because **1** and **2** are used in formation of the undecaprenyl and decaprenyl diphosphates used in bacterial cell wall biosynthesis, as well as in quinone biosynthesis and protein prenylation.^[7, 8, 9] Since this pathway is absent in humans (who use the mevalonate pathway for isoprenoid biosynthesis), both IspG and IspH are of interest as new drug targets.^[10] Both proteins are also present in the plastids of plants where they are involved in quinone,

chlorophyll (phytol) and carotenoid biosynthesis [8, 11] and are, therefore, targets for new herbicides.^[11] How these proteins function has, however, been a mystery for many years since they need to catalyze both electron-transfer reactions (like ferredoxins), as well as the more “chemical” aspect of substrate dehydroxylation.

In this Review we cover 3 main topics: 1) the structures of IspG and IspH; 2) the catalytic mechanisms of IspG and IspH; and 3) the inhibition of IspG and IspH. The results described support a direct role for the [Fe₄S₄] cluster in catalysis as well as inhibition. Specifically, the unique, 4th Fe of the [Fe₄S₄] cluster is involved in formation of “bioorganometallic” π -, η^3 -allyl or ferraooxetane reaction intermediates, and inhibitors also bind to (and in some cases, react with) the [Fe₄S₄] clusters. These results lead not only to unique enzyme-catalyzed reaction mechanisms, but also give clues for the development of novel inhibitors of interest as new drug (and herbicide) leads.

2. Structures of IspG and IspH

2.1 Historical background, and some bioinformatics

In early work, Adam et al. showed that both proteins contained [Fe₄S₄] clusters coordinated to three Cys residues that were found to be essential for catalysis.^[12] This [Fe₄S₄(Cys)₃] coordination motif is the same as that found in aconitase^[4] and suggests that substrates (3 or 4) might coordinate to the unique, 4th Fe, facilitating the electron transfer/reductive dehydroxylation steps. A series of bacterial IspH protein partial sequences are shown in a ClustalW^[13] alignment in Figure 1a with the 3 Cys that are coordinated to the [Fe₄S₄] cluster indicated. To identify other functionally important residues, we used the JPRED3 server^[14] to produce an alignment of 461 IspH sequences from different organisms, then used this alignment as input to the SCORECONS server,^[15] which produces an overall residue “conservation score” ranging from 1.000 (most highly conserved) to 0 (not conserved). We show in Figure 1b the JPRED3/SCORECONS results for some of the top conserved residues in *Aquifex aeolicus* IspH. Besides the three Cys required for [Fe₄S₄] cluster binding, H124, E126, S221 and N223 (H124, E126, S225 and N227 in *E. coli* IspH) were found to have very high conservation scores and these residues are indeed essential for IspH catalysis, based on site-directed mutagenesis results.^[16, 17]

In the case of IspG, there are two different classes of enzyme. In most bacteria, a multiple sequence alignment reveals that there are three conserved cysteine residues (Figure 1c) and two major domains (A, B, Figure 1d) containing numerous conserved residues, with a SCORECONS analysis^[15] indicating that E204 (*A. aeolicus* numbering; 232 in *Thermus thermophilus*) being one of the most essential residues, consistent with mutagenesis results.^[18, 19]

In plants (e.g. *Arabidopsis thaliana*), malaria parasites (e.g. *Plasmodium falciparum*) as well as in several other bacteria (such as *Chlorobium tepidum* and *Chlamydia trachomatis*), a bioinformatics analysis reveals not two but three domains: A, A* and B (Figure 1d).^[19, 20] The A* domains have about the same overall length as the A domains, but there are essentially no conserved amino-acids in the different A* sequences. This strongly suggests a primarily structural as opposed to a more direct, catalytic role, for the A* domain. The

question then arises: what are the three dimensional structures of IspH and of the 2-domain and 3-domain IspGs? Where are the essential residues? How are they involved in catalysis?

2.2. X-ray investigations of IspH structure

The first X-ray crystallographic structure of an IspH was reported by Reikittke et al. for the *Aquifex aeolicus* protein (PDB ID 3DNF),^[21] and was followed shortly after by the structure of the *E. coli* protein (PDB ID 3F7T) by Gräwert et al.^[16] In both cases, it was found that the protein adopted a “trefoil” fold with three α/β domains surrounding a central $[\text{Fe}_3\text{S}_4]$ cluster, Figure 2a. Based on previous work using EPR spectroscopy,^[22] it was suggested that the crystallographically observed $[\text{Fe}_3\text{S}_4]$ cluster was actually an artifact caused by loss of one Fe from a $[\text{Fe}_4\text{S}_4]$ cluster, during crystallization. Computational “reconstitution” of the 4th Fe enabled a substrate ligand-docking investigation in which it was proposed that the substrate **4** bound to the 4th Fe via O-1, forming an alkoxide complex^[21] – the first step in catalysis, as described in more detail below, and indeed in later work, the X-ray crystallographic structure of IspH containing an $[\text{Fe}_4\text{S}_4]$ cluster with bound **4** (PDB ID 3KE8) was obtained by Gräwert et al.^[23] which supports this proposal, as do the results of Mössbauer spectroscopy.^[24] This ligand-bound structure showed a more closed conformation than that found in the absence of the ligand, with the totally conserved E126 being in close proximity to the $[\text{Fe}_4\text{S}_4]$ cluster, as well as to the hydroxyl group of **4**. The diphosphate moiety hydrogen bonded to a series of polar residues including the highly conserved H124, S225 and N227 (H124, S221 and N223 in *A. aeolicus* IspH), as shown in Figure 2b.

2.3. X-ray and EM investigations of IspG structure

Two years after the first reports of the IspH structures, Lee et al. obtained the crystal structure of IspG, from *A. aeolicus* (PDB code 3NOY),^[18] a two domain (AB) IspG, work that was followed soon after by a report by Reikittke et al.^[26] of the structure of IspG from *T. thermophilus* (PDB code 2Y0F), another 2-domain protein. *A. aeolicus* IspG actually crystallizes as a dimer, $(\text{AB})_2$, Figure 3a. The A (N-terminal) domain belongs to the TIM-barrel superfamily^[27] and has closest structural homology to dihydropteroate synthase,^[28] while the B (C-terminal) domain houses the $[\text{Fe}_4\text{S}_4]$ cluster and consists of a fold that is similar to that seen in sulfite reductase^[29] and the ferredoxin domains of nitrite reductase.^[30] One glutamate (E350 in *T. thermophilus*; E307 in *A. aeolicus*) and three Cys residues of the B domain coordinate to the $[\text{Fe}_4\text{S}_4]$ cluster. The distances between the putative active sites in an AB monomer are very large, $\sim 20\text{\AA}$, and both groups proposed that only the dimers would be active since they could adopt a head-to-tail structure (Figures 3a, b), in which the active sites would be made up from the A domain from one chain and the B domain from the second chain; a “hinge-bend” or open/closed motion during catalysis would enable substrate/product ingress/egress. This proposal was recently confirmed by Reikittke et al. by solution of a **3**-bound IspG structure (Figures 3b, c) in which the diphosphate group of **3** clearly binds to the A-domain of one molecule in the dimer while the C-3 OH group is coordinated to the unique 4th Fe in the $[\text{Fe}_4\text{S}_4]$ cluster in the B domain of the second molecule in the dimer (Figure 3c), initiating catalysis.^[31]

To date, there have been no reports of the X-ray crystallographic structures of a 3-domain IspG. However, Liu et al. reported that the results of six different structure-prediction programs all indicated that the A* or “insert” domain in several 3-domain IspGs would also be expected to adopt a TIM barrel fold,^[19] – just as found in the A domains. The results suggested a three-domain A(TIM)-A*(TIM)-B(Fe₄S₄) structure, Figure 1d, in which all the conserved residues are in the A and B domains. This structural proposal then received support from the results of single particle electron tomography, where it can be seen (Figure 3d) that the homology model (for *A. thaliana* IspG) fits well into the electron density observed by electron microscopy.^[19] Thus, the catalytic mechanism for the 2-domain and 3-domain IspGs are expected to be the same, the only difference being that the A* domain plays a primarily structural role in the 3-domain proteins, while in the 2-domain proteins, the A domain plays both a structural as well as a direct role in catalysis.

2.4. Active site iron-sulfur clusters probed by Mössbauer spectroscopy

The electronic structures and ligand coordination of the iron-sulfur cluster at the active sites of IspG and IspH have been directly probed by using ⁵⁷Fe Mössbauer spectroscopy. Seemann et al. first reported a Mössbauer spectroscopic study of ⁵⁷Fe reconstituted oxidized ([Fe₄S₄]²⁺) IspGs from *A. thaliana* (a 3-domain, plant protein, Figure 4a) and *E. coli* (a 2-domain protein, Figure 4b).^[33] Their results clearly showed that both 2 and 3-domain proteins had extremely similar Mössbauer spectra characteristic of [Fe₄S₄] clusters. In particular, there was a 3:1 signal intensity ratio, proposed to arise from 3 tetrahedral-sulfur-coordinated Fe^{2.5+} sites, the 4th site having 3 S together with one non-cysteine (N or O) ligand – consistent with later observations^[31] that this 4th ligand in oxidized IspG arises from a glutamate residue.

With IspH, Mössbauer results for the oxidized protein were rather different to those reported for IspG, in that there were now 3 iron sites, having a 2:1:1 intensity ratio (Figure 4c).^[24, 34] Site 1 was characteristic of tetrahedrally sulfur-coordinated Fe^{2.5+} centers of mixed-valence iron pairs with a delocalized excess electron. Site 2 was characteristic of a high-spin ferric iron, while site 3 was characteristic of a high-spin ferrous iron. When compared to other [Fe₄S₄] proteins having known coordination geometries, it was proposed that oxidized IspH contains 3S and 3N/O ligands^[24] – although the actual nature of the N/O-containing ligands still remains to be determined. More importantly, on binding the substrate **4** to the oxidized protein, the isomer shift of the third component (high-spin Fe²⁺) decreased, from $\delta_3 = 0.89$ mm sec⁻¹ to $\delta_3 = 0.53$ mm sec⁻¹, showing that **4** binds to the unique 4th Fe since the other spectral components did not change (Figure 4d), consistent with the earlier computational predictions.^[21] The same results were obtained with *E. coli* cells overexpressing IspH, strongly supporting the idea that the active site in IspH (in cells) contains an [Fe₄S₄] cluster, not an [Fe₃S₄] cluster.^[24]

These bioinformatics, X-ray crystallographic and Mössbauer spectroscopic investigations led to detailed, but essentially “static” pictures of IspH and IspG structure. To understand how these two enzymes catalyze the 2H⁺/2e⁻ reductions, transient species (i.e. reaction intermediates) need to be trapped, and characterized. Taking advantage of the paramagnetic nature of reduced [Fe₄S₄] clusters, electron paramagnetic resonance spectroscopy has played

an important role in revealing the identities of several reaction intermediates trapped during catalysis, and when combined with the results from X-ray crystallographic, stereochemical and other experimental (and theoretical) studies, lead to detailed mechanistic proposals for both IspH as well as IspG catalysis, and, *seriatim*, to their inhibition.

3. The catalytic mechanism of IspH

How IspH catalyzes the “reductive dehydroxylation” of HMBPP (**4**) has been a mystery for nearly a decade, and seven mechanisms have been proposed, including carbocation, carbanion, and carbon radical reaction intermediates. [16, 22, 35, 36, 37, 38, 39, 40] Recent spectroscopic studies found no evidence for any radical species,[41] and point to a “bio-organometallic” mechanism involving direct iron-carbon interactions during catalysis,[17, 41] as summarized in Scheme 2. Three putative intermediates have been trapped and characterized, leading to reaction mechanism proposals based on EPR/HYSCORE/ENDOR and density functional theory (DFT) calculations; Mössbauer; X-ray crystallographic as well as stereochemical results.

3.1 IspH intermediate I: The alkoxide intermediate

To initiate the catalytic cycle, the substrate **4** first binds to the oxidized cluster ($[\text{Fe}_4\text{S}_4]^{2+}$) of IspH, forming Intermediate I (Scheme 2). As discussed earlier, computational docking,[21] Mössbauer spectroscopy[24, 42] as well as X-ray crystallography[23] all point to an η^1 -alkoxide (or alcoholate) complex being this intermediate. This species may (Scheme 2a), or may not (Scheme 2b), be involved in steady-state catalysis; in the latter scenario, IspH can be reduced by excess reductant right after the formation of products **1** or **2**. Substrate **4** then displaces the product and directly binds to the reduced $[\text{Fe}_4\text{S}_4]^+$ cluster, forming Intermediate II and skipping Intermediate I. Support for this possibility comes from the observation that the product-bound $[\text{Fe}_4\text{S}_4]^+$ cluster was observed in the EPR study of steady-state catalysis when substrate **4** is consumed in the presence of excess reductant (Figure 1 in ref. [17]). More experiments are required in order to determine which scenario is more likely.

3.2 IspH intermediate II: the weak π -complex with the rotated hydroxymethyl group

The original bioorganometallic mechanism proposed that on IspH reduction, the hydroxymethyl group of **4** in Intermediate I rotates away from the $[\text{Fe}_4\text{S}_4]^+$ cluster to interact with E126, a totally conserved residue proposed to serve as a proton donor,[21] forming Intermediate II. This proposal is supported by the results of three experiments. First, rotation of the hydroxymethyl group is in fact now observed in a crystal structure of a wild-type *E. coli* IspH:**4** complex pre-irradiated with X-rays.[43] In this experiment, the IspH:**4** complex was likely photoreduced by X-ray pre-irradiation; the hydroxymethyl group of **4** then dissociated from the unique 4th iron and rotated away from the cluster to hydrogen bond with the diphosphate group of **4** as well as E126 (Figure 5a). Second, an intermediate was trapped by IspH E126Q or E126A mutant (Figure 5b). The ^{17}O -HYSCORE spectrum of this intermediate prepared with $[1\text{-}^{17}\text{O}]\text{-4}$ showed only a ~ 1 MHz ^{17}O hyperfine interaction (Figure 5c). This is much smaller than the ^{17}O hyperfine interactions found in systems containing direct Fe-O bonds such as aconitase, which are characterized by ^{17}O hyperfine

coupling constants in the 8–15 MHz range.^[44, 45] The small ^{17}O hyperfine coupling indicates that the terminal hydroxyl group of **4** does not bind to the reduced cluster ($[\text{Fe}_4\text{S}_4]^+$), consistent with the X-ray pre-irradiated crystal structure of the IspH:**4** complex. The third experiment that supports this critical rotation was reported by Dickschat and coworkers^[46] who used ^2H -isotopic labeling, finding that the observed *E/Z*- ^2H labeling pattern in the IPP product **1** necessitated removal of the 1- CH_2OH group from the cluster with rotation. The rotation is critical from a mechanistic perspective since this step is absent in the radical^[39] and ferroxetane^[47] models (*vide infra*) for IspH catalysis. It should be noted that more work is required to establish the kinetic competency of this intermediate in the wild-type IspH catalyzed reaction, – but clearly computational-docking, EPR as well as the X-ray results all suggest this critical rotation occurs, and E126 is a better proton source compared with T167 proposed in alternative mechanistic proposals.^[39, 40]

The early EPR spectroscopic study^[17] suggested that Intermediate II was a π -complex, not a free radical. The similarity in *g*-tensors of trapped Intermediate II (Figure 5b, $g = [2.124, 1.999, 1.958]$)^[17] to the values observed with an allyl alcohol-bound nitrogenase α -70^{Ala} mutant ($g = [2.123, 1.998, 1.986]$)^[48] suggested the possibility that the alkene substrate **4** formed a similar “ σ/π -complex” to that reported for allyl alcohol bound to the nitrogenase α -70^{Ala} mutant.^[48, 49] In fact, in a comparison with the *g*-tensors of 80 other $[\text{Fe}_4\text{S}_4]$ cluster-containing proteins and model complexes (Figure 5d), it can be seen that the *g*-tensor of Intermediate II clearly clusters with systems that have alkene or alkyne ligands and is characterized by $g_{\text{iso}} > g_{\text{e}}$, (the free electron *g*-value).^[41] It is unlikely that Intermediate II arises from a free radical based on its highly anisotropic *g*-tensor, as well as the results of an ENDOR study which showed 26 and 39 MHz ^{57}Fe hyperfine coupling constants for the two pairs of Fe in the $[\text{Fe}_4\text{S}_4]$ cluster, but very small (~ 1 MHz) ^{13}C hyperfine coupling constants from $[U\text{-}^{13}\text{C}_5]\text{-4}$,^[17] demonstrating that most of the spin density is located on the $[\text{Fe}_4\text{S}_4]$ cluster.

Taken together, all of the results described above indicate that Intermediate II is a π -complex with the hydroxymethyl group rotated away from the reduced cluster ($[\text{Fe}_4\text{S}_4]^+$). Considering that the Fe-C distances ($2.8\text{--}3.3$ Å)^[43] observed in the crystal structure are longer than those observed in classical organometallic π -complexes/metallacycles, together with the fact that the C2-C3 carbons and their attached atoms are essentially planar, that is, are not pyramidalized as in e.g. Zeise’s salt,^[50] Intermediate II is perhaps best described as a weak π or van der Waals complex, and essentially “sets up” **4** in the active site for the next steps in the reaction.

3.3. IspH intermediate III: the η^3 -allyl complex

The terminal hydroxyl group of **4** in Intermediate II interacts with the proton donor E126. In the next catalytic step, this hydroxyl group is protonated and removed as a water molecule, forming Intermediate III. This intermediate has been trapped by adding **4** to one-electron reduced IspH,^[47, 51] as well as by rapid freeze-quenching the reaction of wild type IspH with **4** under steady-state conditions (Figure 6a),^[41] and is characterized by an anisotropic *g*-tensor (e.g. $g = [2.173, 2.013, 1.997]$ for the intermediate trapped with *A. aeolicus* or *P. falciparum* IspH).^[47] More detailed pre-steady state kinetic studies are desirable to confirm

the kinetic competence of this intermediate,^[52] but the kinetics observed under steady state conditions are consistent with the kinetics of the enzyme in the presence of methyl viologen. This intermediate disappeared within 5 seconds when 120 equiv. dithionite, 1 equiv. methyl viologen and 50 equiv. **4** were used,^[41] in agreement with the specific activity of 16.3 $\mu\text{mol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$,^[34] and the k_{cat} value of 9.8 s^{-1} . When methyl viologen was excluded from the reaction with dithionite being the sole reductant, the reaction is three orders of magnitude slower, and this species can be observed for a much longer time, depending on the amount of substrate and reductant added.

The terminal hydroxyl group is not present in this species, as evidenced by the absence of any ^{17}O hyperfine interaction in the HYSCORE spectrum of samples prepared by using $[1\text{-}^{17}\text{O}]\text{-4}$.^[41, 51] The spin density distribution as revealed by the EPR spectroscopic data make it unlikely that Intermediate III is an allyl free radical. Moreover, the continuous-wave EPR spectrum significantly broadened when ^{57}Fe -enriched IspH was used (Figure 6a), indicating that most spin density is located on the $[\text{Fe}_4\text{S}_4]$ cluster. This is consistent with the small ^{13}C and ^2H hyperfine coupling constants observed for Intermediate III, as summarized in Figure 6b:^[41, 47, 51] the ^2H and ^{13}C hyperfine coupling constants are more than seven times (^2H) and fifteen times (^{13}C) smaller than those expected for an allyl radical.^[53]

The nature of Intermediate III is further revealed by its g -tensor, which is characterized by $g_{\text{iso}} > g_{\text{e}}$. This is rather unusual for a $[\text{Fe}_4\text{S}_4]^+$ cluster and is reminiscent of that of an oxidized high-potential iron-sulfur protein (HiPIP, $[\text{Fe}_4\text{S}_4]^{3+}$).^[54] To understand the mechanistic implications of this unusual g -tensor, it is of interest to examine the catalytic mechanism of ferredoxin-thioredoxin reductase (FTR), a well studied $[\text{Fe}_4\text{S}_4]$ enzyme that catalyzes the $2\text{H}^+/2\text{e}^-$ reduction of a disulfide bond.^[55, 56, 57] As with IspH, FTR generates an intermediate with $g_{\text{iso}} > g_{\text{e}}$, ($g = [2.11, 2.00, 1.98]$),^[56] due to a two-electron reduction of the disulfide bond by the reduced iron-sulfur cluster $[\text{Fe}_4\text{S}_4]^+$, forming a HiPIP-like cluster $[\text{Fe}_4\text{S}_4]^{3+}$ and avoiding generation of a thiol free radical.^[56, 57] With this reaction in mind, the HiPIP-like g -tensor of Intermediate III suggests a two-electron reduction of **4** by the reduced iron-sulfur cluster ($[\text{Fe}_4\text{S}_4]^+$) in IspH catalysis, resulting in an allyl anion η^3 -complexed to the HiPIP-like $[\text{Fe}_4\text{S}_4]^{3+}$ cluster.

3.4 Other IspH mechanistic possibilities

Other proposed mechanisms can be categorized into two types: (i) mechanisms involving free radical intermediates, represented by the Birch reduction-like mechanism (Scheme 3),^[39, 40] and (ii) mechanisms involving other bioorganometallic species (Scheme 4).^[47]

With the Birch reduction-like mechanism, there are two difficulties. First, the rotation of the hydroxymethyl group of **4** suggested by computational docking,^[21] EPR spectroscopy,^[41] X-ray crystallography^[43] and confirmed by the isotope-labeling stereochemical study^[46] is absent in the Birch reduction-like mechanism. Instead, it is proposed that the terminal hydroxyl group of **4** is protonated and removed by T167, a much weaker proton donor than E126. Second, the proposed radical species have not been observed in any experiments, and the species that have been observed are not free radicals. Indeed, in quantum chemical calculations,^[51] we found that a $[\text{Fe}_4\text{S}_4]^{2+}$ /radical cluster is less stable than is a $[\text{Fe}_4\text{S}_4]^{3+}$ /allyl anion, that is, an internal electron transfer occurs during geometry optimization.

To explain the nature of the $g_1 = 2.17$ reaction intermediate (Intermediate III in our mechanism), another model featuring a ferraoxetane (**5**, Scheme 4) has been proposed.^[47] This structure is very reminiscent of that we proposed earlier for Intermediate X in IspG catalysis (*vide infra*);^[19, 58, 59, 60] however, the involvement of the ferraoxetane **5** in the IspH reaction is inconsistent with several experimental observations, the major one being the absence of any ^{17}O hyperfine interaction in samples prepared with $[1-^{17}\text{O}]\text{-4}$,^[41] ruling out the possibility of an Fe-O bond. In addition, there is no evidence for any direct bonding interaction between the apical Fe and C2: the ^{13}C hyperfine coupling constants for C1, C2 and C3 are all small (1.8 – 3.2 MHz; Figure 6b) and can be reproduced by DFT calculations on a model $\eta^3\text{-allyl}$ complex,^[51] while the Fe-C2 ^{13}C hyperfine coupling in the IspG ferraoxetane is ≈ 17 MHz.^[59] Other difficulties with this model include the absence of the critical rotation of the hydroxymethyl group, its inability to explain the formation of product **2**, as well as the reaction with a fluorine substrate analog which does not form any Fe-F bond.^[41, 61]

4. The catalytic mechanism of IspG

In a very early mechanistic study of IspG catalysis, the three highly conserved Cys residues were identified and mechanisms similar to that of vitamin K epoxyquinone reductase or ribonucleotide reductase were proposed.^[62] It was then found that IspG was a $[\text{Fe}_4\text{S}_4]$ enzyme with three conserved Cys residues coordinated to a $[\text{Fe}_4\text{S}_4]$ cluster.^[22, 63] Thereafter, several mechanisms involving carbocations, carbanions, an epoxide and carbon radical reaction intermediates were proposed (Scheme 5).^[36, 63, 64, 65]

The first three mechanisms shown in Scheme 5 all share a carbocation as the first reaction intermediate. The importance of such an intermediate was later supported by isotope-exchange experiments using a $[^{13}\text{C}2, ^{18}\text{O}]\text{-labeled}$ substrate MEcPP (**6**, Scheme 6).^[66] Based on the different chemical shifts of C2 when bonded to either ^{16}O or ^{18}O , it was demonstrated that on addition of IspG and in the absence of any reductant, **6** can be converted to **7**, suggesting opening of the *cyclo*-diphosphate ring and transient formation of the carbocationic species **8**, Scheme 6. The observed rate of conversion was, however, 63 times lower than the k_{cat} of IspG catalyzing the reductive dehydroxylation of **3**, due possibly to the restricted rotation of the diphosphate group in **8**.^[66]

An alternative mechanism was proposed by Rohdich *et al.*^[36] in which formation of an epoxide intermediate initiates catalysis (Scheme 5d). This proposal was based on previous observations that an epoxide could be reduced to an ethylene by a synthetic $[\text{Fe}_4\text{S}_4]$ cluster^[67] and is supported by the fact that the epoxide **9** is indeed an IspG substrate (Scheme 7) with a k_{cat} (20.1 min^{-1}) comparable to that of the natural substrate **3** (23.7 min^{-1}).^[68] Furthermore, **9** and **3** form the same paramagnetic intermediate (Intermediate X, *vide infra*) when reacted with IspG, as identified by EPR and ^1H -ENDOR spectroscopy.^[58] These results, do not, however, establish the intermediacy of **9** in the reductive dehydroxylation of **3** by IspG. Efforts to identify the formation of **9** from **3** under either oxidized or reduced conditions were not successful, whereas the reverse reaction, the conversion of **9** to **3** catalyzed by oxidized IspG ($k_{\text{cat}} \sim 2.0 \text{ min}^{-1}$) was observed.^[69] Thus, it

is likely that **9** is not normally involved in catalysis. Rather, both **9** as well as **3** can form the same reaction intermediate, Intermediate X, that is then converted to **4** (HMBPP).

The next reaction intermediate in the previously proposed mechanisms (Scheme 5) is a carbon free radical, but as with IspH, so far no radical species have been reported. A paramagnetic species characterized by $g = [2.087, 2.019, 2.000]$ (Intermediate X, Figure 7a) is, however, observed by EPR in the freeze-quenched reaction of IspG with **3**,^[70] or with **9**.^[58] It is unlikely to be a free radical, though, because its EPR spectrum broadens significantly when trapped with ⁵⁷Fe-enriched IspG (Figure 7a), indicating that a large amount of spin density is located on the [Fe₄S₄] cluster. More compelling evidence for the nature of Intermediate X comes from extensive hyperfine coupling tensor measurements which suggest that “X” is a ferraooxetane, an organometallic species containing both Fe-C and Fe-O bonds, as described in the following. But why a ferraooxetane? Such a species would appear to be highly strained, but that is not necessarily a problem for a reaction intermediate. Moreover, the *cyclo*-diphosphate ring opening would provide a possible binding site (C2) to the unique 4th iron of the [Fe₄S₄]⁺ cluster to form an Fe-C bond.

Evidence for an Fe-C bond comes from ¹³C hyperfine coupling measurements on Intermediate X. A large ¹³C hyperfine coupling was observed in the HYSCORE spectrum of X trapped with [*U*-¹³C₆]-labeled **3**, with a hyperfine coupling tensor $A(^{13}\text{C}) = [14.5, 12.0, 26.5]$ MHz (Figure 7b).^[59] The 17.7 MHz isotropic hyperfine coupling constant $a_{\text{iso}}(^{13}\text{C})$ is significantly larger than the 3.7 and 1.1 MHz $a_{\text{iso}}(^{13}\text{C})$ observed in the nitrogenase:allyl alcohol complex, where the allyl alcohol is proposed to directly bind to the FeMo cofactor, forming a metallacycle with one of the Fe.^[48] It is comparable to the 17.1 MHz $a_{\text{iso}}(^{13}\text{C})$ of ¹³CO observed in CO inhibited [FeFe] hydrogenase, where CO directly binds to the H_{ox} cluster.^[71] The strongly coupled carbon in X was specifically assigned to C2 (the quaternary carbon) by using [2,3-¹³C₂]- and [1,3,4-¹³C₃]-labeled **3**; its neighboring carbon (C3) has a much weaker hyperfine coupling constant $a_{\text{iso}}(^{13}\text{C}) = 3.0$ MHz, and all the other carbons have $a_{\text{iso}}(^{13}\text{C}) < 1$ MHz.^[58, 59]

Evidence for an Fe-O bond comes from ¹⁷O HYSCORE studies of X prepared by using specifically ¹⁷O-labeled substrates. Because the chemical synthesis of ¹⁷O-labeled **9** is much easier than that of ¹⁷O-labeled **3**, and because **9** forms the same Intermediate X as does **3**, [1-¹⁷O] and [2,3-¹⁷O]-labeled **9** were prepared and used to prepare “X”. With [1-¹⁷O]-labeled **3**, only a very small ¹⁷O hyperfine coupling (~0.15 MHz, by simulation, Figure 7c) was observed, whereas Intermediate X prepared from [2,3-¹⁷O]-**9** exhibited a large ¹⁷O hyperfine coupling (~8 MHz, Figure 7d). The ~8 MHz hyperfine coupling constant is comparable to that observed when H_x¹⁷O ($A(^{17}\text{O}) = 8 \sim 12$ MHz)^[44] or ¹⁷O-labeled substrates or substrate analogs ($A(^{17}\text{O}) = 9 \sim 15$ MHz)^[45] are bonded to the unique iron (Fe_a) of aconitase. The ¹⁷O-HYSCORE results thus indicate that the 3-OH group, but not the 1-OH group, of **3** directly binds to the unique 4th iron of the [Fe₄S₄] cluster in X. This conclusion is strongly supported by the crystal structure of IspG complexed with **3**^[31] (Figure 3c), in which there is an Fe-O3 bond (in the oxidized state of the iron-sulfur cluster).

The results of ¹³C and ¹⁷O hyperfine coupling measurements indicate bonding of C2 and O3 to the [Fe₄S₄] cluster in Intermediate X, narrowing down the possible structures for this

species. The most likely candidate appears to be the ferraioxetane **10**, shown in Scheme 8. **10** may look unusual, but several metallaoxatanes are known as stable species.^[72, 73] Moreover, in the case of iron interacting with oxirane, the 1,2-ferraioxetane has been observed using matrix isolation,^[74] and on warming, the ferraioxetane undergoes a [2+2] dissociation to ethylene and FeO.^[74]

The results of ³¹P and ¹H ENDOR spectroscopy are also consistent with assignment of X to the ferraioxetane **10**. The ³¹P hyperfine coupling tensor has been determined to be either an isotropic dominated tensor ($A = [0.21, 0.09, 0.05]$ MHz) or a dipolar dominated tensor ($A = [0.22, -0.11, -0.09]$ MHz).^[60] In either case, the very small hyperfine couplings indicate that the diphosphate group does not bind to the iron-sulfur cluster. Using a point-dipole model, a minimum distance of 6.6 Å from the phosphorus nucleus to the unique 4th iron was estimated, based on the dipolar-dominated tensor.^[60] The ¹H hyperfine coupling constants of all the protons derived from **3** were determined by ENDOR spectroscopy and selective ²H isotopic labeling.^[59] The largest ¹H hyperfine coupling ($A(^1\text{H}) = [14, 11, 11]$ MHz)^[60] in Intermediate X was assigned to one of the protons of the C2' methyl group; all the other protons, including the other two protons of the C2' methyl group, have much smaller hyperfine couplings.^[59] The small anisotropy of the $A(^1\text{H}) = [14, 11, 11]$ MHz hyperfine tensor is consistent with this proton being in the second coordination sphere of the [Fe₄S₄] cluster, having a weak dipolar interaction with the paramagnetic center. The large difference in the hyperfine coupling constants for the three protons in the C2' methyl group was well reproduced by DFT calculations on a model ferraioxetane **11** (*vide infra*),^[19] further supporting this structural assignment.

A DFT calculation on a model ferraioxetane [Fe₄S₄(SMe)₃(-C(CH₂OH)(CH₃)-CH(CH₂OH)-O)]²⁻ (**11**, Scheme 8)^[19] gave a not unreasonable set of predictions of the experimentally measured hyperfine coupling constants. In particular, the large difference in the hyperfine coupling constants for the three protons of the C2' methyl group was reproduced, and was found to arise from the dependence of the hyperfine coupling constants on the H-C-C-Fe dihedral angles. The calculated large coupling ($A_i(^1\text{H}) = 9.1$ MHz) was in reasonable accord with the experimental result (12 MHz), and arose from the *trans* (Fe-C-C-H torsion angle = 172°) proton, while the smaller hyperfine couplings originate from the *gauche* (+/-) protons with geometry optimized torsion angles of 52, -67°.^[19] Similar dihedral angle dependences of hyperfine coupling constants have been observed for the Cβ protons of cysteine ligands to [Fe₄S₄]⁺ clusters,^[75] and are similar to the well-known observation of large ³J *trans* scalar couplings in NMR spectroscopy.

There are two other structures that have been proposed for Intermediate X (Scheme 10)^[60]: the carbanions **12** and **13**. Structure **12** is unlikely because a carbanion would not be expected to be stable (since CH groups have pK_a values of ~40), and more importantly this structure would be inconsistent with the large hyperfine coupling constant observed for C2. Structure **13** is likewise unlikely: it is not an oxallyl (which might be stable) as a result of the protonation of O, and ²H₃ is not exchanged during isoprenoid biosynthesis.^[76]

When combined with the results of X-ray crystallography and site-directed mutagenesis, the IspG mechanism shown in Scheme 9 is suggested. The first step is coordination of O3 (the

oxygen to be removed as a water molecule) to the unique 4th iron of the oxidized iron-sulfur cluster ($[\text{Fe}_4\text{S}_4]^{2+}$), forming the alkoxide/alcoholate complex (Intermediate I) observed in the crystal structure of IspG co-crystallized with **3**.^[31] This reaction is likely catalyzed by E232 – the most highly conserved non-Cys residue in IspG based on a SCORECONS analysis.^[15] The opening of the *cyclo*-diphosphate ring and formation of the carbocation (Intermediate II) is reversible once **3** binds to the oxidized iron-sulfur cluster, as evidenced by the exchange of ^{18}O of $[\text{2-}^{13}\text{C}, ^{18}\text{O}]\text{-3}$ in the presence of oxidized IspG.^[66] Once an electron is accepted from a reductant, a ferroxetane (X) forms. Considering the HiPIP-like *g*-tensor with $g_{\text{iso}} = 2.035$, it seems likely that the one-electron reduced iron-sulfur cluster ($[\text{Fe}_4\text{S}_4]^+$) performs an internal two-electron reduction of **3** to form the ferroxetane, similar to that proposed for FTR and now, IspH catalysis. The ferroxetane then dissociates to the final product **4** and oxidized IspG by accepting one more electron and two protons. Which protein residues are involved in these reactions is not known, but among the top 6 most conserved residues (E232, S202, N346, R302, S262 and N112, in *T. thermophilus*), E232 seems a likely candidate given its acidity and close proximity to O3 in the IspG:3 crystal structure.

5. Inhibitors targeting IspG and IspH

5.1. Substrate analogs as inhibitors

Substrate analogs that bind to IspG or IspH but cannot turn over could be potent inhibitors of interest as new anti-infective drug leads, and herbicides. Replacing the diphosphate group with isosteres such as carbamates (**14**), *N*-acyl-*N'*-oxy sulfamates (**15**), or aminosulfonyl carbamates (**16**), Scheme 10, resulted in only weak inhibitory effects against IspH or IspG.^[77] The substitution of the diphosphate group also abolished the ability of these compounds to activate human V γ 9V δ 2 T cells where **4** is a ~30 pM activator.^[78, 79]

In contrast, substituting the terminal hydroxyl group with a thiol (**17**) or an amino group (**18**), Scheme 10, led to potent IspH inhibitors with IC₅₀ values of 0.21 μM and 0.15 μM , respectively, against IspH.^[42, 80] Both inhibitors were not turned over by IspH (unlike the fluoro-analogs^[40, 41]) and bind reversibly with the terminal thiol or amino groups coordinating to the unique 4th iron, forming complexes similar to the alkoxide complex observed with the natural substrate, **4**. **18** was found to be a slow binding inhibitor, possibly due to the deprotonation step required to bind to the $[\text{Fe}_4\text{S}_4]$ cluster.^[80] Such binding modes were established by using Mössbauer spectroscopy (Figures 8a, b) and DFT calculations,^[42] and were later confirmed by X-ray crystallography (Figures 8c, PDB code 4H4E, and Figure 8d, PDB code 4H4D).^[61] With **18**, an additional conformation in which the aminomethyl group rotates away from the $[\text{Fe}_4\text{S}_4]$ cluster was observed, due perhaps to photoreduction of the iron-sulfur cluster in the X-ray beam.^[61] At present, it is not clear whether the inhibition of enzyme catalysis is due to binding to the oxidized clusters, the reduced clusters, or both.

5.2. Rational inhibitor design based on the IspH catalytic mechanism

The bioorganometallic mechanism of IspH catalysis has led to the “rational” design of potent inhibitors that target both IspH as well as IspG. The “rational” aspect is that, with IspH, we are proposing formation of π -complexes with olefins and allyl anions in which

case it might be expected that other species – acetylenes – would also bind to the $[\text{Fe}_4\text{S}_4]$ cluster. There are in fact several precedents for the formation of organometallic species between synthetic $[\text{Fe}_4\text{S}_4]$ clusters and alkynes. For example, McMillan *et al.*^[81] proposed the formation of an organometallic species containing a side-on acetylene unit to account for the *cis*-reduction of acetylene to ethylene by the reduced cluster $[\text{Fe}_4\text{S}_4(\text{SPh})_4]^{3-}$.^[81] More direct evidence for the formation of π -complexes comes from the significant shifts of the acetylenic vibrational Raman spectra of acetylene when bound to a reduced $[\text{Fe}_4\text{S}_4]$ cluster.^[82] The binding and inhibition of IspH (and IspG) by propargyl alcohol (**19**) as well as a series of alkyne diphosphates (**20** - **23**) and their isoelectronic analogs (**24** and **25**), shown in Scheme 11, was thus investigated.^[17, 58, 83] As expected, **19** binds to reduced IspH, as evidenced by EPR spectroscopic data which indicate large *g*-value changes (Figure 9a), although **19** has poor inhibitory activity against IspH. This improves upon addition of a diphosphate group with the resulting propargyl diphosphate (**20**) having an IC_{50} of 6.7 μM against *A. aeolicus* IspH.^[17] Like **19**, **20** changes the EPR spectrum of IspH (Figure 9b), and an $A(^{13}\text{C}) \sim 6$ MHz was observed by ENDOR spectroscopy when $[U\text{-}^{13}\text{C}_3]$ -**19** or **20** were used (Figure 9c). Only a very small ^{31}P hyperfine coupling constant (~ 0.3 MHz) was observed, indicating the diphosphate group does not bind to the $[\text{Fe}_4\text{S}_4]^+$ cluster (Figure 9d). The best acetylenic inhibitor found to date was **21** with an IC_{50} of 0.45 μM ($K_i \sim 60$ nM) against *A. aeolicus* IspH.^[83] The isoelectronic cyano diphosphates **24** and **25** both had much weaker inhibitory effects when compared with their alkyne diphosphate counterparts.

The alkyne diphosphates also turn out to be potent inhibitors against IspG. For example, **20** is a competitive IspG inhibitor with an $\text{IC}_{50} \sim 750$ nM ($K_i \sim 330$ nM).^[19, 58] The EPR spectrum of **20** bound to IspG is similar to that observed when **20** binds to IspH, and the ENDOR spectrum exhibits a significant ($A(^{13}\text{C}) \sim 7$ MHz) hyperfine interaction when $[U\text{-}^{13}\text{C}_3]$ -**20** is added to reduced IspG, consistent with formation of a π -complex.^[58]

5.3. Inhibitors discovered from compound library screening

A third class of IspH inhibitors, pyridine diphosphates (**26** and **27**, Scheme 14), were discovered by screening a library of diphosphates and bisphosphonates previously developed as prenyl synthase inhibitors.^[83] These compounds bind directly to the $[\text{Fe}_4\text{S}_4]^+$ cluster of *A. aeolicus* IspH via the nitrogen of the pyridine moiety, as evidenced by the change in the EPR spectrum on ligand addition (Figure 10a), and more importantly, by the presence of a large ^{14}N hyperfine coupling observed in HYSCORE spectra of *A. aeolicus* IspH bound to **26** (Figure 10b) or **27**.^[84] The ^{14}N hyperfine coupling tensor of the pyridine nitrogen was determined to be $A = [6.2, 7.6, 8.4]$ MHz, and the nuclear quadrupole coupling constant $e^2qQ/h = 3.0$ MHz; both are comparable to the values found in other systems containing known Fe-N bonds.^[84] However, these pyridine diphosphates were only weak inhibitors against *E. coli* IspH ($\text{IC}_{50} = 0.5$ mM for **26**).

6. IspH is also an acetylene hydratase

To learn more about how these acetylenic inhibitors might bind to IspH, X-ray crystallographic structure determinations of **20**-**22** bound to oxidized IspH were initiated as a prelude to structure determination of their binding to reduced IspH. The results revealed

that **20-22** bind to oxidized IspH quite differently to the binding mode proposed for the reduced enzyme. More significantly, an unexpected hydratase activity of IspH was discovered in which oxidized IspH converts **21** to an aldehyde and **22** to a ketone (Scheme 13).^[85]

The crystal structure of **20** bound to oxidized IspH (Figure 11a, PDB code 3URK) shows that the acetylenic carbons of **20** are 3.4 and 3.6 Å away from the unique 4th iron. In addition, there is a water molecule (or, in principle, a hydroxide ion) bound to the [Fe₄S₄] cluster with a Fe-O bond length of 2.1 Å, essentially the same Fe-O distance as is found when **4** is bound to oxidized IspH.^[21, 23]

The X-ray crystal structures of IspH in complex with **21** or **22** were even more surprising, since they showed that both ligands had undergone a hydration reaction. The crystal structure of IspH co-crystallized with **21** indicated formation of the η^1 -enolate complex **28** by anti-Markovnikov addition, and again there was a 2.0 Å Fe-O bond length (Figure 11b, PDB code 3UTC). This complex actually turns over, with the aldehyde **29** being detected.^[85] In the case of **22**, again the crystal structure revealed a chemical reaction of the acetylene group (Figure 11c, PDB code 3UTD), but rather than forming an aldehyde, the X-ray results now indicated Markovnikov addition, with formation of the ketone, **30**.^[85]

The reactions catalyzed by IspH are summarized in Scheme 13. All of the substrates contain a diphosphate group which binds to the conserved diphosphate binding site^[16, 38] and position C4 of the ligands close to the unique 4th Fe of the oxidized cluster ([Fe₄S₄]²⁺). Thus: with **20**, the C₃ side-chain is too short to interact with the unique 4th Fe (Scheme 13a), but with the C₄ species **21**, OH (presumably from a cluster-bound water, as seen in the structure of IspH:**20**) adds to C4 *via* anti-Markovnikov addition to form the η^1 -enolate **28**, which is then released as the aldehyde **29** (Scheme 13c). With a C₅ side chain (**22**), OH once again adds at C4, but now, protonation must be at C5 -Markovnikov addition - resulting in the formation of ketone **30** (Scheme 13d). This non-redox hydratase reaction is one of the well-known functions catalyzed by several [Fe₄S₄] proteins (such as aconitase) and in the case of *E. coli* fumarase A (FumA), the enzyme is also capable of catalyzing the hydration of an acetylene, acetylene dicarboxylate,^[5, 86, 87] to oxaloacetate. Thus, although IspH is optimized by Nature to function as a 2H⁺/2e⁻ deoxygenase, when an appropriate ligand is positioned in the right location, it can also exhibit hydratase activity –one of the intrinsic activities of other oxidized [Fe₄S₄] clusters.

7. Summary and Outlook

The structures and mechanisms of action of the [Fe₄S₄] cluster-containing enzymes IspG and IspH have been of considerable interest for many years because they produce the C₅ “building blocks”, IPP (**1**) and DMAPP (**2**), of the largest class of small organic molecules on Earth: the terpenes. Understanding their structures, mechanisms of action – and inhibition – could lead to new drugs as well as new herbicides. IspG and IspH both catalyze 2H⁺/2e⁻ reductions, most likely via bioorganometallic reaction intermediates. In IspG catalysis, we propose that a ferroxetane intermediate containing Fe-C and Fe-O bonds is involved; in IspH catalysis, a weak π -complex and an allyl η^3 -complex are involved. The mechanisms

proposed represent the first examples of bioorganometallic catalytic mechanisms of [Fe₄S₄] enzymes, which then led to the design of the first potent IspH and IspG inhibitors, alkyne diphosphates. Additional inhibitors have been discovered by compound library screening, and by making substrate analogs. These novel inhibitors open up potentially new routes to anti-infective drug design targeting [Fe₄S₄]-containing proteins in the MEP isoprenoid biosynthesis pathway.

There are, however, still many unanswered questions. From a structural perspective, key questions include: what are the exact structures of the three-domain IspGs? Which ligands are bound to the cluster in oxidized IspH? Can one obtain crystal structures of the reaction intermediates? From a functional perspective, several important aspects still need to be addressed. First, although high activity has been achieved by using dithionite and artificial electron mediators,^[34] the native redox system remains elusive. How relevant, then, are these model redox systems to the situation found in cells? Second, there is a need for more detailed pre-steady state kinetic studies on the proposed reaction intermediates. Third, the proposed HiPIP-like intermediates need to be prepared in high yields for further characterizations (e.g. by Mössbauer spectroscopy), to test their HiPIP-like nature. From the perspective of inhibitor design: Can one obtain potent IspG/IspH inhibitors that are active in cells, and *in vivo*? Would an IspH-only inhibitor be a good or a bad thing? It could certainly kill the targeted pathogens and the concomitant accumulation of **4** would activate $\gamma\delta$ T cells (containing the V γ 2V δ 2 T-cell receptor) – but this could be too much of a good thing since it could lead to sepsis.^[88] So, IspG or combined IspH + IspG inhibitors might be required, for safety and efficacy.

The results on oxidized IspH also suggest an exciting *nexus* between the [Fe₄S₄] clusters in IspH and other [Fe₄S₄] proteins that are drug/herbicide targets. For example, the fact that IspH hydrates acetylenes in the same way as does FumA is of interest since FumA in malaria parasites is a drug target.^[89] Likewise, the enzymes dihydroxyacid dehydratase (DHAD)^[90] and isopropylmalate isomerase (IPMI)^[91] are targets for inhibiting branched chain amino-acid biosynthesis in tuberculosis bacteria (inside macrophages) and are also of interest as herbicide targets, and diverse inhibitors have been reported.^[92] Some may also inhibit IspH and IspG, while the leads that inhibit IspH and IspG may provide new ideas for DHAD and IPMI inhibitors—all targeting [Fe₄S₄] clusters with a unique 4th iron.

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Biographies

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Figure 1.

Bioinformatics analysis of IspH and IspG. a) Multiple sequence alignment for IspH. b) Some of the top conserved residues (besides the conserved cysteines). The first column shows the residue numbers in *A. aeolicus* IspH; *E. coli* IspH numberings are shown in parentheses. The second column shows the conservation scores calculated by SCORECONS.^[15] The third and fourth columns show the mutants made, and their activities. c) Multiple sequence alignment for IspG. d) Simplified view of A, A* and B domain organizations.

Figure 2.

X-ray crystal structure of IspH. a) Ligand-free *A. aeolicus* IspH, which shows a trefoil fold with a [Fe₃S₄] cluster at the center (PDB code 3DNF).^[21] b) *E. coli* IspH with bound **4**, showing that **4** forms an alkoxide complex with the [Fe₄S₄] cluster (PDB code 3KE8).^[23] Some of the key residues at the active site are shown. Figures were prepared using VMD.^[25]

Figure 3.

Structures of IspGs. a) Ligand-free *T. thermophilus* IspG (PDB code 2Y0F).^[26] b) *T. thermophilus* IspG with bound **3** (PDB code 4G9P).^[31] c) **3** binds to the [Fe₄S₄] cluster via O3 forming an alkoxide complex; the highly conserved residue E232 is 3.7 Å away from O3.^[31] d) A model of the three-domain IspG from *A. thaliana* docked into a 20-Å electron density map created by single particle electron tomography of *A. thaliana* IspG.^[19] a)–c) were prepared using VMD.^[32] d) was adapted from ref. [19] with permission.

Figure 4.

Mössbauer spectra of IspG and IspH. a) Mössbauer spectrum of 356 μM ^{57}Fe -reconstituted *A. thaliana* IspG obtained at $T=77$ K;^[33] b) Mössbauer spectrum of 413 μM ^{57}Fe -reconstituted *E. coli* IspG obtained at $T=77$ K.^[33] Adapted from ref. [33] with kind permission from Springer Science and Business Media. c) Mössbauer spectrum of *E. coli* IspH obtained at $T=77$ K;^[24] d) Mössbauer spectrum of *E. coli* IspH with **4** bound obtained at $T=77$ K.^[24] Adapted from ref. [24] with permission, Copyright (2009) American Chemical Society.

Figure 5.

Intermediate II of IspH catalysis. a) The hydroxymethyl group rotated Intermediate II was observed together with the alkoxide complex with crystals pre-irradiated with X-ray. Reprinted from ref. [43], Copyright (2011), with permission from Elsevier. b) The EPR spectrum of ligand-free *A. aeolicus* IspH (top) and Intermediate II trapped with an E126A mutant. Adapted from ref. [17] with permission. c) HYSCORE spectrum of Intermediate II trapped with $[1-^{17}\text{O}]\text{-4}$; d) Plot of g_{iso} vs. g for 80 iron-sulfur containing systems. Adapted from ref. [41] with permission, Copyright (2012) American Chemical society.

Figure 6.

Intermediate III of IspH catalysis. a) X-band EPR spectrum of Intermediate III trapped with natural abundance or ⁵⁷Fe-enriched wild-type E. coli IspH. Adapted from ref [41] with permission, Copyright (2012) American Chemical society. b) Hyperfine coupling constants of ligand atoms in Intermediate III. The $a_{iso}(^2\text{H})$ for the deuteron on C1 was determined in ref. [47], and others were determined in refs. [41, 51].

Figure 7.

EPR spectroscopic characterization of Intermediate X of *E. coli* IspG. a) continuous-wave EPR of Intermediate X. The red line is the intermediate trapped with ^{57}Fe -enriched IspG. b) – d) HYSCORE spectrum of Intermediate X prepared with b) $[\text{U}-^{13}\text{C}]\text{-3c}$ c) $[1-^{17}\text{O}]\text{-9}$ and d) $[2,3-^{17}\text{O}]\text{-9}$. Adapted from refs. [19], [58, 59] with permission.

Figure 8.

Inhibitors **17** and **18** binding to *E. coli* IspH. a) and b) show Mössbauer spectra of **17** and **18** binding to IspH, respectively, collected at 77 K, $B = 0$ T (upper traces) and 5 K, $B = 5$ T applied perpendicular to the γ beam (bottom traces). Adapted from ref [42] with permission, Copyright (2013) American Chemical society. c) and d) show crystal structures of **17** and **18** complexed to IspH, respectively. Adapted from ref. [61] with permission, Copyright © 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Figure 9.

Propargyl alcohol or propargyl diphosphate binding to *A. aeolicus* IspH. a) and b), X-band EPR spectra of **19** or **20** binding to *A. aeolicus* IspH. c) and d), X-band ^{13}C and ^{31}P ENDOR spectra of **20** binding to *A. aeolicus* IspH. Adapted from ref. [17] with permission.

Figure 10.

X-band EPR and HYSCORE spectra of IspH with the pyridine inhibitor **26**. (a) EPR spectra of ligand-free *A. aeolicus* IspH (top) and *A. aeolicus* IspH + **26** (bottom). (b) HYSCORE spectrum of reconstituted *A. aeolicus* IspH + **26**. Adapted from ref. [84] with permission, Copyright (2011) American Chemical society.

Figure 11.

X-ray crystal structures of alkyne diphosphate or their converted products binding to *E. coli* IspH. (a) **20** binds to *E. coli* IspH. (b) **21** is converted to the enolate **28** with *E. coli* IspH. (c) **22** is converted to the ketone **30** with *E. coli* IspH. Figures were prepared by VMD. [32]

Scheme 1.
Reactions catalyzed by IspG and IspH.

Scheme 2.

The bioorganometallic mechanism of IspH catalysis. OPP represents the diphosphate group.

Scheme 3.

The proposed Birch reduction-like mechanism of IspH catalysis.^[39, 40] OPP represents the diphosphate group. Adapted from ref. [41] with permission, copyright (2012) American Chemical society.

The ferroxetane mechanism for IspH catalysis.^[47] Adapted from ref. [41] and [47] with permission, Copyright (2012) American Chemical society.

Scheme 5.

Proposed reaction mechanisms of IspG catalysis. (a), cation $\rightarrow$ radical mechanism of Kollas *et al.*^[64] (b), cation $\rightarrow$ radical $\rightarrow$ cation radical mechanism of Seemann *et al.*^[63] (c), cation $\rightarrow$ radical $\rightarrow$ anion mechanism of Brandt *et al.*^[65] (d), oxirane $\rightarrow$ radical mechanism of Rohdich *et al.*^[36]

Scheme 6.
Isotopic exchange of **3** catalyzed by oxidized IspG

Scheme 7.
Reactions of epoxide **9** with IspG.

Scheme 8.

Proposed structures for Intermediate X. OPP represents the diphosphate group.

Scheme 9.

Proposed mechanism of IspG catalysis based on EPR, HYSCORE, ENDOR, DFT and x-ray results. Proton delivery could also occur in both of the last two steps.

Scheme 10.
Substrate analogs as IspH inhibitors.

Scheme 11. Alkyne and cyano diphosphate inhibitors investigated.^[83]

Scheme 12.
Pyridine diphosphate inhibitors against *A. aeolicus* IspH.^[83]

Scheme 13.
Summary of IspH reactions.