Supporting Information

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SI Text

Supplementary Methods. ***Materials.*** All chemicals were purchased from Sigma-Aldrich and were used as provided. ^{57}Fe metal was purchased from American Elements. The plasmid encoding wild-type IspH from *Aquifex aeolicus* was the generous gift of Dr. Hassan Jomaa and Dr. Jochen Wiesner. The plasmid encoding the *E. coli* IspG was kindly provided by Dr. Pinghua Liu. XL2-Blue ultracompetent cells and BL-21(DE3) competent cells were purchased from Stratagene.

Site-directed mutagenesis. To trap a reactive intermediate in IspH catalysis, we elected to use the active site E126A mutant of *Aquifex aeolicus*, originally proposed (1) as being critical for catalysis and as confirmed in the *E. coli* protein (2). Point mutation was carried out using the Stratagene QuickChange II Site-Directed Mutagenesis kit. The forward and reverse primer sets for the E126A mutant were 5′-GAGAAAAATCACCCGGCAGT-GATCGGTACGCTG-3′ and 5′-CAGCGTACCGAT-CACTGCCGGGTGATTTTTCTC-3′, respectively. Underlined base-pairs represent the site of mutagenesis. The construct was first denatured at 95 °C for 5 min, then amplified for 16 cycles (denaturation at 95 °C for 30 sec; annealing at 55 °C for 30 sec; elongation at 72 °C for 5 min), followed by additional elongation at 72 °C for 10 min, and finally cooled down to 4 °C, using PfuUltra high-fidelity DNA polymerase (Stratagene). After PCR, the product was digested with DpnI at 37 °C overnight to remove parental methylated and hemimethylated DNA. 5 μL of the digest was subsequently transformed into XL2-Blue Ultracompetent cells (Stratagene) and spread onto LB plates with 100 μg/mL ampicillin until colonies appeared. Individual colonies were grown in 5 mL of LB medium with 100 μg/mL ampicillin. The plasmids from an overnight culture were then extracted using the Qiagen Miniprep. The plasmids were sequenced using an ABI 3730XL capillary sequencer and the plasmid with the correct sequence chosen for expression and purification.

IspH protein purification. BL-21(DE3) cells producing either wild-type or E126A mutant IspH from *A. aeolicus* were grown in LB media supplemented with 150 mg/L ampicillin at 37 °C until the OD_{600} reached 0.6. Cells were then induced with 200 μg/L anhydrotetracycline, then grown at 20 °C for 15 h. Cells were harvested by centrifugation (9000 rpm, 8 min, 4 °C) and were kept at −80 °C until use. Cell pellets were resuspended and lysed in B-PER (Thermo Scientific) protein extraction reagent for one h at 4 °C, then centrifuged at 200,000 rpm at 4 °C for 15 min. The supernatant was applied to a Ni-NTA column equilibrated with 5 mM imidazole in a pH 8.0 buffer containing 50 mM Tris•HCl and 150 mM NaCl. After washing with 20 mM imidazole, protein was eluted with 100 mM imidazole. Fractions were collected and dialyzed in pH 8.0 buffer containing 50 mM Tris•HCl, 150 mM NaCl, 5% glycerol, and 1mM DTT, 4 times. The purified protein was flash-frozen in liquid nitrogen and stored at −80 °C until use.

Protein reconstitution. IspH protein (either wild-type or the E126A mutant) as isolated had a very small peak at 410 nm (A_{280}/A_{410} < 0.02), so was reconstituted for further studies. Before reconstitution, protein was transferred into a Coy Vinyl Anaerobic Chamber after being degassed on a Schlenk line. All the following steps were performed inside the anaerobic chamber with an oxygen level <2 ppm. In a typical reconstitution experiment, 10 mM DTT and approximately 0.5 mg of elemental sulfur were added to 3 mL 0.6 mM protein solution in a pH 8.0 buffer containing 50 mM Tris•HCl, 150 mM NaCl, and 5% glycerol. After stirring for 1.5 h, $FeCl_3$ was then added slowly to 6 equivalents from a 30 mM stock solution. After 3 h, an aliquot of the solution was centrifuged and a UV-VIS spectrum recorded. If the $A_{410\,nm}/A_{280\,nm}$ ratio was ≥0.38, the protein was then desalted by passing through a PD10 column. If the ratio was <0.38, more DTT, elemental sulfur and $FeCl_3$ were added and incubation (with stirring) continued for a longer time (typically 2 h) until the ratio was approximately 0.38. The reconstituted protein was then concentrated by ultrafiltration, and the protein concentration determined with a BioRad Protein Assay kit. For the ^{57}Fe enriched sample, ^{57}Fe metal was dissolved in HCl in air, then after pH adjustment, was purged with nitrogen gas and used for reconstitution as described above.

Enzyme inhibition assays. All assays were performed anaerobically at room temperature according to Altincicek et. al. (3), with minor modifications. To a pH 8.0 buffer solution containing 50 mM Tris-HCl, 150 mM NaCl, and 5% glycerol, sodium dithionite was added to 0.4 mM, methyl viologen was added to 2 mM, and IspH was added to 72 nM. For enzyme assays, various amounts HMBPP were added and the reactions were monitored at 732 nm. Initial velocities were fit by using the Michaelis–Menten equation with OriginPro 8 (OriginLab Corporation). For inhibition assays, various concentrations of inhibitors were added and incubated for 10 min, prior to addition of 34 μM HMBPP. Initial velocities at different inhibitor concentrations were then plotted as dose-response curves, and were fit to the following equation, from which IC_{50} value were determined

$$y = \frac{1}{1 + (\frac{x}{IC_{50}})^{\text{slope}}}$$

where y is the fraction inhibition and x is the inhibitor concentration. K_i values were then deduced from the IC_{50} value by using the Cheng–Prusoff equation (4)

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_M}}$$

where $[S]$ is the HMBPP concentration, and K_M is the Michaelis constant.

EPR and ENDOR spectroscopy. Samples for EPR spectroscopy were typically 0.3 mM in IspH and 3 mM in ligand (except for propargyl diphosphate which was 12 mM). Samples were reduced either by adding 20 equivalents of sodium dithionite and incubating for 5 min, or by photoreduction in the presence of 300 μM 5-deazaflavin, 10 mM ammonium oxalate, and 5 mM DTT, using illumination from a 300 W LCD projector at approximately 1 cm for 2.5 h. For EPR/ENDOR spectroscopy, glycerol was added to 42.5% (v/v). EPR samples were frozen in liquid nitrogen after reduction. EPR spectra were collected at X-band using a Varian E-122 spectrometer together with an Air Product helium cryostat. Data acquisition parameters were typically field center = 3250 G; field sweep = 800 G; modulation = 100 kHz; modulation amplitude = 5 G; time constant = 32 ms; 60 sec per scan; 8 sec between each scan; and temperature = 15 K. EPR spectra were simulated by using the "EasySpin" program (5). Samples for ENDOR spectroscopy were typically

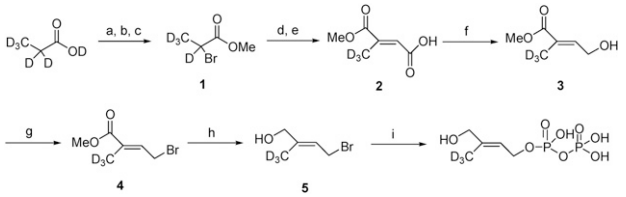
1.2 mM in IspH and 12 mM in ligands (except 48 mM for propargyl diphosphate). Samples were reduced by adding 20 equivalents of sodium dithionite, and were frozen in liquid nitrogen 5 min after sodium dithionite addition. Pulsed ENDOR spectra were obtained on a Bruker ElexSys E-580-10 FT-EPR X-band EPR spectrometer using an ENI A 300RF amplifier and an Oxford Instruments CF935 cryostat at 15 K (8 K for unliganded [^{57}Fe]-wt IspH). Davies pulsed ENDOR experiments were carried out for ^{57}Fe samples and the IspH + propargyl alcohol sample, using a three pulse scheme (π_{mw}-T-$\pi/2_{mw}$-τ-π_{mw}-τ-echo, π_{rf} was applied during T) (6) with the excitation field set to correspond to g_2. ^{57}Fe Davies ENDOR spectra were collected with either $\pi/2_{mw} = 12$ ns (hard pulse) or $\pi/2_{mw} = 48$ ns (soft pulse) excitation. With the soft pulse scheme, both proton and ^{57}Fe signals are detected, while with the hard pulse scheme, only the ^{57}Fe signals are detected (because protons have smaller hyperfine couplings and are suppressed). The soft pulse scheme was also used for aquiring ^{1}H ENDOR spectra of IspH + propargyl alcohol. For other samples, Mims pulsed ENDOR was employed using a three pulse scheme ($\pi/2_{mw}$-τ-$\pi/2_{mw}$-T-$\pi/2_{mw}$-τ-echo, $\pi/2_{mw} = 16$ ns and π_{rf} was applied during T) with the excitation field set to correspond to g_2. τ-averaging (32 spectra at 8 ns step) was used to reduce the blind spots that arise from the τ-dependent oscillations.

Docking calculation. For docking calculations, the IspH target protein (PDB: 3F7T) was prepared using the protein preparation wizard in Maestro 8.0 (7). Water from the active site region was removed, but the diphosphate was kept in the active site. The Fe_3S_4 cluster was reconstituted computationally to form the Fe_4S_4 species as described previously (1) and hydrogen atoms were added to the protein. Hydrogen bonds were optimized to default values and an energy minimization in MacroModel 9.5 (8) performed only on the protein hydrogens, using default parameters. A receptor grid large enough to encompass all crystallographically observed binding sites was then generated from the prepared target protein. Geometry optimized ligands were docked using Glide (9) extraprecision (XP) mode and no other constraints were applied. In some instances, we also used the MMFF94 forcefield (10) to provide additional geometry optimization.

Synthetic aspects. All reagents used were purchased from Aldrich. The purities of all compounds produced were routinely monitored by using ^{1}H and ^{31}P NMR spectroscopy at 400 or 500 MHz on Varian Unity spectrometers. Cellulose TLC plates were visualized by using iodine or a sulfosalicylic acid-ferric chloride stain (11).

[4-^{2}H]-HMBPP was synthesized according to the literature (12). ^{1}H NMR (400 MHz, D_2O) δ 1.69 (s, 3H), 3.99 (s, 1H), 4.51 (t, J = 7.1 Hz, 2H), 5.64 (t, J = 6.8 Hz, 1H); ^{31}P NMR (162 MHz, D_2O) δ −10.25 (d, J = 22.0 Hz), −6.53 (d, J = 22.0 Hz).

[5-$^2H_3$]-(E)-4-hydroxy-3-methyl-but-2-enyl diphosphate. To synthesize [5-$^2H_3$]-HMBPP, we modified a reported procedure using propionic acid-d_6 as starting material to introduce the three deuterium atoms at the C5-methyl position (13).



(a) $SOCl_2$, CCl_4, 65 °C, 1 h; (b) NBS, 48% HBr, CCl_4, 85 °C, 3 h; (c) MeOH, pyridine, $CHCl_3$, 0 °C, 1 h, 52% (above 3 steps); (d) PPh_3, CH_3CN, 65 °C, 12 h; (e) OHC-CO_2H monohydrate, iPr_2NEt, CH_3CN, 0 °C, 62% (above 2 steps); (f) BH_3, THF, −10 °C, 55%; (g) PBr_3, CCl_4, 61%; (h) DIBAL, THF, −78 °C, 1 h, 67%; (i) $(n\text{-}Bu_4N)_3HP_2O_7$, CH_3CN, 41%.

[2, 3, 3, 3-$^2H_4$]-Methyl 2-bromopropionate (1). A mixture containing 2.40 g (0.03 mol) propionic acid-d_6, 5 mL of dry CCl_4, and 4.38 mL (0.06 mol) of $SOCl_2$ was heated at 65 °C for 1 h until the acid was converted to the acid chloride (14, 15). N-Bromosuccinimide (6.41 g, 0.036 mol) in anhydrous CCl_4 (6 mL), and 48% hydrobromic acid (2 drops) were added to the acid chloride. The temperature was raised to 85 °C and the mixture heated for 3 h. After cooling, the mixture was filtered to remove the precipitated succinimide, and the filtrate was concentrated under reduced pressure. The residue was slowly added to a solution of methanol (1.28 g, 0.04 mmol) and pyridine (2.40 g, 0.03 mol) in chloroform (10 mL) at 0 °C (ice bath). After stirring for 1 h the reaction mixture was successively washed with water, sulfuric acid (10%), and saturated aqueous $NaHCO_3$ and dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue purified by flash chromatography on silica gel (hexane: EtOAc = 4:1) to yield 2.65 g (52%) of a pale yellow oil. ^{1}H NMR(400 MHz, D_2O) δ 3.79 (s, 3H).

[2-$^2H_3$]-2-Methylmonomethylfumarate (2). A mixture containing [2, 3, 3, 3-$^2H_4$]-methyl 2-bromopropionate (**1**) (1.71 g, 0.01 mol), dry CH_3CN (25 mL), and triphenylphosphine (2.62 g, 0.01 mol) was heated at 65 °C for 12 h. After cooling at 0 °C, diisopropylethylamine (1.29 g, 0.01 mol) and glyoxylic acid monohydrate (0.92 g, 0.01 mol) in dry CH_3CN (5 mL) were added to the reaction mixture. The solution was further stirred at 0 °C for 2 h and slowly allowed to warm to room temperature overnight. Half of the solvent was removed under reduced pressure. The resulting solution was washed with saturated aqueous $NaHCO_3$ (3 × 30 mL). The combined aqueous layers were extracted with EtOAc (2 × 10 mL), acidified (pH 1–2) at 0 °C with concentrated HCl and extracted with EtOAc (3 × 30 mL). The combined organic layers were evaporated to dryness, yielding a pale yellow solid (0.91 g, 62%), which was used for the next reaction without further purification.^{1}H NMR (500 MHz, D_2O) δ 3.84 (s, 3H), 6.82 (s, 2H), 11.92 (br, 1H).

[2-$^2H_3$] Methyl (E)-4-hydroxy-2-methyl-but-2-enoate (3). A 1 M solution of borane–tetrahydrofuran complex (4.0 mL, 4 mmol) was added dropwise to a solution of **2** (0.59 g, 4 mmol) in dry THF (25 mL) at −10 °C. The reaction mixture was slowly allowed to warm to room temperature overnight. The reaction was quenched by dropwise addition of 50% aqueous acetic acid (1.8 mL). After removal of the solvent, saturated aqueous $NaHCO_3$ was dropwise added at 0 °C. The aqueous layer was extracted with EtOAc. The extracts were washed with saturated aqueous $NaHCO_3$ and the combined aqueous layer was again extracted with EtOAc. The combined organic layers were combined and concentrated to afford a pale yellow oil (0.29 g, 55%), which was used for the next step without further purification. ^{1}H NMR (400 MHz, D_2O) δ 3.75 (s, 3H), 4.34 (d, J = 6.4 Hz, 2H), 6.82 (t, J = 6.4 Hz, 1H).

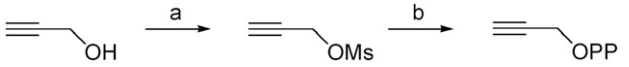
[2-$^2H_3$] Methyl (E)-4-bromo-2-methyl-but-2-enoate (4). To a solution of **3** (266 mg, 2 mmol) in CCl_4 (5 mL) was added PBr_3 (180 mg, 0.67 mmol) at 0 °C. After stirring at room temperature for 1 h, the reaction mixture was quenched with saturated aqueous $NaHCO_3$. The organic layer was washed with water and the resulting aqueous layers were then extracted with pentane. The organic layers were concentrated to a pale yellow oil (239 mg, 61%), which was used for the next step without further

purification. ^{1}H NMR (400 MHz, D_2O) δ 3.75 (s, 3H), 4.01 (d, J = 9.0 Hz, 2H), 6.91 (t, J = 9.0 Hz, 1H).

[2-$^2H_3$]-(E)-4-bromo-2-methylbut-2-en-1-ol (5). A 1 M solution of DIBAL in hexane (2.5 mL, 2.5 mmol) was added dropwise via syringe to a stirred solution of **4** (196 mg, 1 mmol) in dry THF (5 mL) at −78° C. After being stirred for 1 h, the reaction mixture was quenched by addition of 50% aqueous acetic acid (3–4 drops). The resulting insoluble material was filtered through Celite and the precipitate was washed with acetone (3 × 20 mL). The solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel (hexane: EtOAc = 2:1) to yield 114 mg (67%) of a pale yellow oil. ^{1}H NMR (400 MHz, D_2O) δ 4.04 (d, J = 8.0 Hz, 2H), 4.08 (s, 2H), 5.82 (t, J = 8.0 Hz, 1H).

[5-$^2H_3$]-(E)-4-hydroxy-3-methyl-but-2-enyl diphosphate. [2-^{2}H$_3$]-(E)-4-bromo-2-methylbut-2-en-1-ol (**5**) (67 mg, 0.4 mmol) was added dropwise to a stirred solution of 0.90 g (1 mmol) tris(tetra-n-butylammonium) hydrogen pyrophosphate in CH_3CN (4 mL) at 0 ° C, the reaction mixture was slowly allowed to warm to room temperature over 2 h and solvent was removed at reduced pressure. The residue was dissolved in 1 mL of cation-exchange buffer (49:1 (v/v) 25 mM NH_4HCO_3/2-propanol) and passed over 90 mequiv of Dowex AG50W-X8(100-200 mesh, ammonium form) cation-exchanged resin preequilibrated with two column volumes of the same buffer. The product was eluted with two column volumes of the same buffer, flash-frozen, and lyophilized. The resulting powder was dissolved in 1 mL of 50 mM NH_4HCO_3. 2-Propanol/CH_3CN (1:*l* (v/v), 2 mL) was added, and the mixture was mixed on a vortex mixer and centrifuged for 5 min at 2000 rpm. The supernatant was decanted. This procedure was repeated three times, and the supernatants were combined. After removal of the solvent and lyophilization, a white solid was obtained. Flash chromatography on a cellulose column (53:47 (v/v) 2-propanol/50 mM NH_4HCO_3) yielded 51 mg (41%) of a white solid. ^{1}H NMR (500 MHz, D_2O) δ 3.99 (s, 2H), 4.51 (t, J = 7.1 Hz, 2H), 5.64 (t, J = 6.8 Hz, 1H); ^{31}P NMR (162 MHz, D_2O) δ −9.38 (d, J = 22.0 Hz), −6.66 (d, J = 22.0 Hz).

Prop-2-ynyl diphosphate (9).



(a) MsCl, Et_3N, 0 °C; (b) tris (tetra-n-butylammonium) hydrogen diphosphate, MeCN, −20 °C.

Propargyl alcohol was converted into its mesylate by reaction with methanesulfonyl chloride (16). Propargyl methanesulfonate (134 mg, 1 mmol) in CH_3CN (0.5 mL) was added dropwise to a stirred solution of 2.70 g (3.0 mmol) tris (tetra-n-butylammonium) hydrogen diphosphate in CH_3CN (5 mL) at −20 °C, then the reaction mixture was slowly allowed to warm to room temperature over 2 h and solvent removed under reduced pressure. The residue was dissolved in 1 mL of cation-exchange buffer (49:1 (v/v) 25 mM NH_4HCO_3/2-propanol) and passed over 90 mequiv of Dowex AG50W-X8 (100-200 mesh, ammonium form) cation-exchange resin preequilibrated with two column volumes of the same buffer. The product was eluted with two column volumes of the same buffer, flash-frozen, then lyophilized. The resulting powder was dissolved in 3 mL of 50 mM NH_4HCO_3, 2-propanol/CH_3CN (1:*l* (v/v), 7 mL) was added, and the mixture vortexed, then centrifuged for 5 min at 2000 rpm. The supernatant was decanted. This procedure was repeated three times, and the supernatants were combined. After removal of the solvent and lyophilization, a white solid was obtained. Flash chromatography using a cellulose column (4:1:2.4 (v/v/v) 2-propanol/CH_3CN/50 mM NH_4HCO_3) yielded 47 mg (18%) of a white solid. ^{1}H NMR (400 MHz, D_2O) δ 2.68 (s, 1H), 4.38 (d, $J_{H,P}$ = 9.2 Hz, 2H); ^{31}P NMR (162 MHz, D_2O) δ −10.10 (d, J = 20.7 Hz), −7.67 (d, J = 20.7 Hz).

[$^{13}C_3$]-prop-2-ynyl diphosphate ([u-^{13}C]-9). [^{13}C$_3$]-Propargyl alcohol (Cambridge Isotopes) was converted into its mesylate by reaction with methanesulfonyl chloride (17). Following the procedure described for prop-2-ynyl diphosphate, 30 mg of [^{13}C$_3$]-labeled propargyl methanesulfonate was converted to 11 mg (19%) of [^{13}C$_3$]-prop-2-ynyl diphosphate. ^{1}H NMR (400 MHz, D_2O) δ 2.68 (dd, $^1J_{H,C}$ = 246.8 Hz, $^2J_{H,C}$ = 55.6 Hz, 1H), 4.38 (dt, $^1J_{H,C}$ = 160 Hz , J = 8.0 Hz, 2H); ^{31}P NMR (162 MHz, D_2O) δ −9.80 − 9.67 (m), −5.85 (d, J = 20.7 Hz); ^{13}C NMR (100 MHz, D_2O) δ 53.71 (ddd, $^1J_{C1,C2}$ = 73.6 Hz, $^2J_{C1,C3}$ = 15.2 Hz, $^2J_{C1,P}$ = 3.8 Hz), 75.11 (dd, $^1J_{C3,C2}$ = 171.5 Hz, $^2J_{C3,C1}$ = 15.2 Hz), 80.45 (ddd, $^1J_{C2,C3}$ = 170 Hz, $J_{C2,C1}$ = 74.3 Hz, $^3J_{C2,P}$ = 11.0 Hz).

[u-$^{13}C_5$] (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate. [u-^{13}C]-HMBPP was prepared from [u-^{13}C]-MEcPP, which was produced by growth of *Corynebacterium ammoniagenes* on [u-^{13}C]-glucose under oxidative stress conditions, basically according to the procotol described by Santos et al. (18) *C. ammoniagenes* (ATCC 6872) was cultured aerobically in a peptone (10 g/L), yeast extract (3 g/L), NaCl (5 g/L) medium at 30 °C with shaking at 225 rpm. When the cells reached late log phase (OD at λ = 600 nm ~ 1.4), the medium was supplemented with benzyl viologen to a final concentration of 50 mg/L, and [u-^{13}C]-glucose to 2.0 g/L. Growth was allowed to continue for 18 h, and the cells were then harvested by centrifugation. The cell pellets (from 1 L growth media) were extracted with 7:3 (v/v) ethanol:water three times, with 40 mL of solvent each time. After rotary evaporation to remove solvent the crude extract was dissolved in 30 mL H_2O, loaded onto a QAE-Sephadex column preequilibrated with 0.05 M NH_4OAc buffer (pH = 7), washed with approximately 100 mL of the same buffer, then eluted with a linear gradient of 0.3–1.5 M NH_4OAc. The desired product eluted at approximately 0.8 M NH_4OAc. The fractions were pooled and lyophilized, and MEcPP detected by using a GcpE activity assay (19) and ^{13}C and ^{31}P-NMR spectroscopy (20). The MEcPP-containing fractions were further purified by cellulose column chromatography, with acetonitrile: isopropyl alcohol: 1% NH_4HCO_3 (aq) 4:2:1 and 1:2:1 as eluents. The 1:2:1 solvent fractions were pooled and lyophilized. The MEcPP product was transferred into a Coy Vinyl Anaerobic chamber, then placed into a Wilmad LabGlass screw-cap NMR tube. *E. coli* GcpE and sodium dithionite were added and the reaction monitored by ^{13}C-NMR spectroscopy. When the reaction was complete, the GcpE enzyme was removed by ultrafiltration using a Microcon. *E. coli* IspG for MEcPP biosynthesis was grown under the same condition as for the *A. aeolicus* IspH. The protein was purified by using strep-tag affinity chromatography (21). The as-purified protein was then reconstituted using the same method as used for reconstituting *A. aeolicus* IspH.

5-deazaflavin. 5-deazaflavin for photoreduction was synthesized by boiling a 1:1 (molar ratio) mixture of 2-aminobenzaldehyde and barbituric acid in water, for one h (22).

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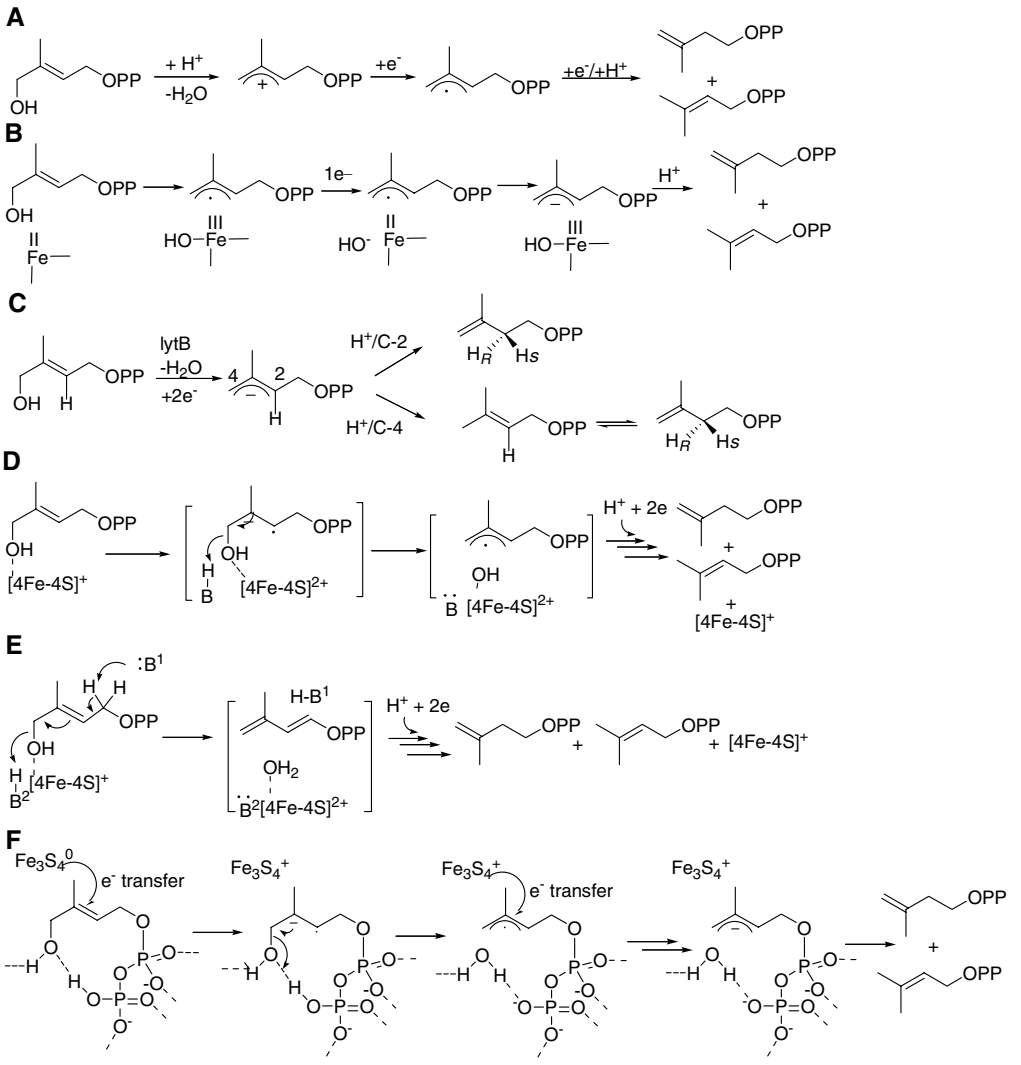

Fig. S1. Previously proposed mechanisms of action of IspH.

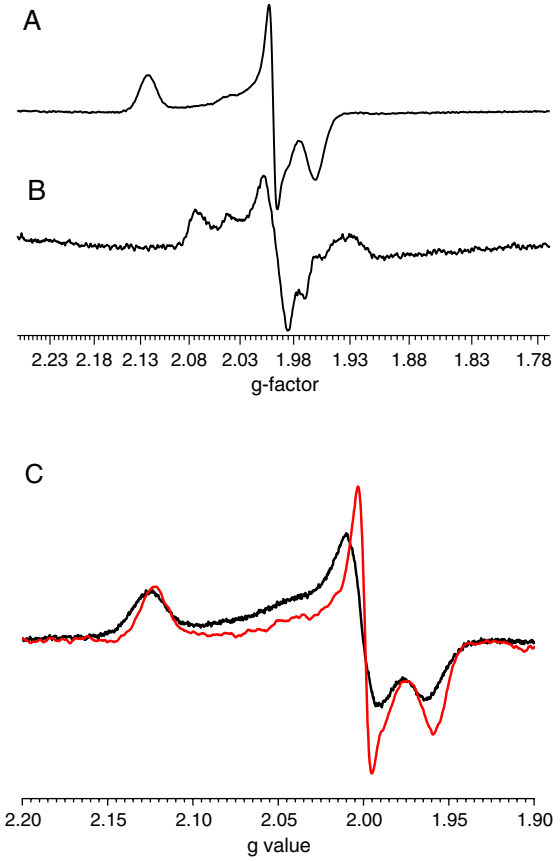

Fig. S2. EPR spectra of samples reduced by photoreduction plus effect of ^{57}Fe on linewidth. (*A*) E126A IspH + HMBPP, photoreduced. (*B*) Wild-type IspH + IPP, photoreduced. (*C*) E126A IspH + HMBPP (*Red*), u-^{57}Fe labeled sample (*Black*).