Published in final edited form as:

J Am Chem Soc. 2011 June 8; 133(22): 8400–8403. doi:10.1021/ja200763a.

An ENDOR and HYSCORE Investigation of a Reaction Intermediate in IspG (GcpE) Catalysis

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Abstract

IspG is a 4Fe-4S protein that carries out an essential reduction step in isoprenoid biosynthesis. Using electron-nuclear double resonance (ENDOR) and hyperfine sublevel correlation (HYSCORE) spectroscopies on labeled samples, we specifically assign the hyperfine interactions in a reaction intermediate. These results help clarify the nature of the reaction intermediate, supporting a direct interaction between the unique 4th Fe in the cluster and the C2 and O3 of the ligand.

Keywords

Isoprenoid; non-mevalonate; IspG (GcpE); HYSCORE; ENDOR

We report here spectroscopic results that help clarify the nature of a key reaction intermediate in isoprenoid biosynthesis. Isoprenoids are the most abundant small molecules on Earth.¹ They are typically made by condensing the C₅ diphosphates dimethylallyl diphosphate (DMAPP, **1**) and isopentenyl diphosphate (IPP, **2**) to form C₁₀, C₁₅, and C₂₀ diphosphates, the precursors of di-, tri-, and tetra-terpenes. DMAPP and IPP are synthesized in two main pathways: the mevalonate pathway² and the methylerythritol phosphate pathway.³ In the latter, the last two steps are catalyzed by unusual 4Fe-4S cluster-containing proteins, IspG (also called GcpE) and IspH (also called LytB). These catalyze the conversion of 2-*C*-methylerythritol-*cyclo*-2,4-diphosphate (MEcPP, **3**) to *E*-1-hydroxy-2'-methyl-but-2-enyl-4-diphosphate (HMBPP, **4**)^{4–6} and thence, to DMAPP and IPP^{7, 8}:

The structure and mechanism of action of both IspG as well as IspH have been of interest for many years. In recent work it was shown that in IspH, HMBPP binds to a unique 4th Fe in the 4Fe-4S cluster, and is then deoxygenated to form an allyl species which is converted to DMAPP/IPP.^{9–12} The mechanism of action of IspG is more complex, and there have been several proposals involving cationic, radical, anionic as well as oxirane

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Supporting Information Available: details on protein production and purification, ENDOR/HYSCORE sample preparation, compound syntheses, and Supporting Information figures are reported in are reported in Supporting Information available free of charge via the Internet at <http://pubs.acs.org>.

intermediates.^{5, 6, 13, 14} The oxirane hypothesis is attractive since oxiranes (epoxides) are known, in model systems, to be converted to alkenes by reduced 4Fe-4S clusters.¹⁵ It is also known that the kinetics of the MEcPP → product as well as the HMBPP-epoxide → product reactions are quite similar.¹⁶ However, this might simply indicate that both MEcPP as well as HMBPP-epoxide form the same reactive intermediate “X”, with the rate-determining step involving breakdown of “X”,¹⁷ consistent with the observation that the electron paramagnetic resonance (EPR) spectrum of the previously known reaction intermediate¹⁸ “X” formed on addition of MEcPP to IspG is indistinguishable from that formed on addition of HMBPP-epoxide.¹⁹ However, the structure of “X” is not known. Here, we discuss the likely structure of “X” based on electron-nuclear double resonance (ENDOR) and hyperfine sublevel correlation (HYSCORE) spectroscopies with ²H and ¹³C -labeled compounds.

Several possible structures for “X” have been proposed recently, including the ferroxetanes (**5,6**),^{19, 20} a protonated ferroxetane (**7**),²⁰ and two carbanions (**8, 9**).²⁰

The ENDOR spectrum of “X” exhibits a single large ¹H hyperfine interaction with a coupling constant (*A*) of ~11 MHz,¹⁹ and more recent measurements have yielded a hyperfine tensor having $A_{ii}({}^1\text{H}) = [14, 11, 11]$ MHz, $a_{\text{iso}} = 12$ MHz.²⁰ This proton signal originates from the MEcPP/HMBPP-epoxide substrates, since it is absent in the ENDOR spectrum of IspG + [u-²H] MEcPP, which exhibits the corresponding ²H signal with *A* = 1.7 MHz (Figure 1a),¹⁹ but the origin of this peak has been unclear. To assign the proton/deuteron signals in “X”, we used four specifically-deuterated HMBPP-epoxides: **10, 11, 12** and **13**.

The reaction intermediate “X” prepared using **10** shows the 1.7 MHz ²H resonance in its X-band Mims ENDOR spectrum (Figure 1b), consistent with the Davies ENDOR spectrum which shows the disappearance of the $a_{\text{iso}} = 12$ MHz proton signal (Figure 1g). Clearly then, this ¹H ENDOR signal arises from one or more protons in the C2' methyl group. Interestingly, in addition to the 1.7 MHz ²H resonance, an *A*~0.37 MHz resonance is also apparent in the Mims ENDOR spectrum (Figure 1b), suggesting nonequivalence of the three methyl protons/deuterons. The three non-equivalent C2' methyl deuteron signals of “X” prepared using **10** are better resolved in Q-band field-dependent ENDOR spectra, as seen in Figure 2, and these spectra can be well simulated with 3 sets of hyperfine couplings in addition to an $e^2qQ/h \sim 165$ kHz... These results indicate that the C2' methyl group is essentially static at 20 K (and at 2 K²⁰ since the line shapes of the 12 MHz proton ENDOR signals are the same at these two temperatures).

The reaction intermediates “X” prepared using **11, 12**, and **13** show ²H resonances with small hyperfine couplings (< 0.5 MHz) in their Mims ENDOR spectra (Figure 1c-e). These signals, together with those from **10**, contribute to the broad, *A*~0.4 MHz resonances seen with [u-²H] MEcPP (Figure 1a), which can be well reproduced by adding the ²H Mims ENDOR spectra of “X” prepared using **10, 11, 12**, and **13** (Figure 1f).

We next consider the ¹³C HYSCORE assignments of the carbons in the reaction intermediate “X”. The HYSCORE spectrum of “X” prepared by using *E. coli* IspG and [u-¹³C] MEcPP (**14**) exhibits three sets of ¹³C signals (Figure 3a): one has a relatively large (~17 MHz) hyperfine coupling, the second a small (~3 MHz) coupling, the third a very small (≤ 1 MHz) coupling, consistent with previous results obtained using *T. thermophilus* IspG.¹⁹ To begin to specifically assign these signals, we obtained HYSCORE spectra using [1,3,4-¹³C₃]-labeled MEcPP (**15**) (Figure 3b) and [2,3-¹³C₂]-labeled MEcPP (**16**) (Figure 3c). The ~17 MHz hyperfine coupling is absent in the [1,3,4-¹³C₃]-labeled sample (Figure 3b), but is present in the [2,3-¹³C₂]-labeled sample (Figure 3c), indicating that this strongly coupled ¹³C signal arises from the quaternary carbon, C2. The results of simulations of

HYSCORE spectra taken at different magnetic field strengths (Figure S1) and different τ -values (Figure S2) indicate that the hyperfine tensor (A_{ii}) of C2 is [14.5, 12.0, 26.5] MHz, with an isotropic hyperfine coupling constant a_{iso} ($^{13}\text{C}2$) of 17.7 MHz.

The ~ 3 MHz ^{13}C signal arises from C3, since it is present in the $[2,3-^{13}\text{C}_2]$ -labeled (**16**) sample (Figure 3c). We also conclude that C3 is the only carbon that contributes to this 3 MHz ^{13}C signal, based on the following: First, **14**, **15** and **16** all give the same line-shapes and peak positions in their ^{13}C HYSCORE spectra for the ~ 3 MHz signals (Figures 3). Second, the ~ 3 MHz ^{13}C HYSCORE signal from samples prepared using **14** and **15** taken at different magnetic field strengths can be well simulated using just a single carbon, having $A_{ii}(^{13}\text{C}3) = [1.8, 2.0, 5.1]$ MHz, $a_{iso}(^{13}\text{C}3) = 3.0$ MHz (Figures S1, S3). Third, the ~ 3 MHz ^{13}C HYSCORE signals of samples prepared using **14** and **15** vary in the same manner with changes in τ -values (Figure S4), and can be well simulated using a single carbon with the hyperfine values given above (Figure S2).

These results suggest that, of all the structures proposed so far, **6** or **7** are the most favored candidates for “X”, for the following reasons: First, the assignment of the $a_{iso} = 12$ MHz hyperfine coupling to a proton in the methyl group is consistent with these models since the $A_{ii}(^1\text{H}) = [14, 11, 11]$ MHz is close to isotropic, indicating a long range interaction. Second, the hyperfine coupling tensor of C2 ($A_{ii}(^{13}\text{C}2) = [14.5, 12.0, 26.5]$ MHz) is highly anisotropic, indicating a strong dipole-dipole interaction with the paramagnetic center, consistent with (see below) an Fe-C bond (**6** or **7**). The observed $^{13}\text{C}2$ hyperfine coupling in “X” is also close to that seen for ^{13}CO directly bonded to one of the irons in the H-cluster in the $\text{H}_{ox}\text{-CO}$ state of a [FeFe] hydrogenase, where $A_{ii}(^{13}\text{CO}) = [19.2, 16.6, 15.6]$ MHz and $a_{iso}(^{13}\text{CO}) = 17.1$ MHz.²¹ In addition, the 17.7 MHz $a_{iso}(^{13}\text{C}2)$ in “X” is much smaller than the 43.8 MHz $a_{iso}(^{13}\text{C})$ found in a formaldehyde-inhibited xanthine oxidase, in which the formaldehyde carbon is two bonds away from the Mo center.²² This 43.8 MHz hyperfine coupling arises from a “trans-annular hyperfine interaction”, and is in good accord with the results of DFT calculations ($a_{iso}(^{13}\text{C}) \approx 47.9$ MHz).²² However, in a structure containing a single Mo-C bond, the same DFT methods yielded an $A_{ii}(^{13}\text{C}) = [23.2, 13.4, 11.7]$ MHz, $a_{iso}(^{13}\text{C}) = 16.1$ MHz, very close to the $^{13}\text{C}2$ hyperfine coupling results found with the reaction intermediate “X” in IspG. These comparisons suggest a direct interaction of Fe with C2 in “X” (**6** or **7**), rather than the large trans-annular hyperfine interaction (**5**) seen in the Mo-containing system, whose square-pyramidal geometry enables a large metal-carbon orbital overlap.

However, **6** and **7** cannot be easily distinguished. A 3.7 MHz ^1H hyperfine coupling is seen on $^2\text{H}_2\text{O}$ exchange,²⁰ but this 3.7 MHz ^1H signal might be from either the protonated ferraooxetane (**7**), or from a proton that is hydrogen-bonded to the iron-sulfur cluster. The former seems less likely since there is no precedent for such a species, and the coupling observed is rather small. As to the other possibilities for “X” that have recently been considered: as noted, **8** is unlikely because a carbanion would not be expected to be stable (since CH groups have pK_a values of ≈ 40 , so the carbanion would be rapidly protonated). Structure **9** is likewise unlikely since not only it is not an oxoallyl (which might be stable), due to the O being protonated, but $^2\text{H}3$ is not exchanged during isoprenoid biosynthesis.²³

Overall, the results presented above are of general interest since they provide new insights into the mechanism of action of IspG, an unusual reductase containing two distinct structural domains.^{24, 25} The results of ^2H and ^{13}C labeling together with spectroscopic/simulation studies on the reaction intermediate “X” enable assignments of the three largest hyperfine couplings seen previously to one $\text{H}2'$ ($a_{iso} = 12$ MHz), C3 ($a_{iso} = 3.0$ MHz) and C2 ($a_{iso} = 17.7$ MHz). The latter value is very similar to that found previously for Fe-C in a hydrogenase,²¹ and to that computed for a Mo-C bond in a xanthine oxidase model,²² both

of which have a_{iso} of ~16–17 MHz, supporting an assignment to a structure containing a metal-carbon bond, e.g. **6**. This involvement of Fe-C bonding is very reminiscent of that found in IspH, in which ~2.6–2.7 Å Fe-C distances are seen,¹² considerably shorter than the combined ~3.6 Å Fe, C van der Waals radii.²⁶ Taken together with the x-ray crystallographic and modeling results,^{24, 25} it thus appears that after initial docking to the triose phosphate isomerase (TIM) barrel in IspG, MEcPP first ionizes; the 4Fe-4S cluster domain then bends over to interact with the first intermediate bound to the TIM barrel, which is then reduced, forming the relatively stable intermediate “X”, **6** or **7**, which bridges both domains, opening up the intriguing possibility of designing inhibitors-drug leads-that may also bridge both domains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Pinghua Liu for providing his IspG expression systems, Dennis Dean for providing his isc protein expression system (pDB1282); and Wolfgang Eisenreich and Victoria Illarionova for providing [1,3,4-¹³C₃]-MEcPP. This work was supported by the United States Public Health Service (NIH grants GM065307 and AI0074233, to EO), and the Hans-Fischer Gesellschaft, Munich (WE, VI). WW was supported by a Predoctoral Fellowship from the American Heart Association, Midwest Affiliate (Award 10PRE4430022). ENDOR/HYSCORE experiments were made possible by equipment grants from NIH (grants S10RR023614 and S10RR025438), NSF (CHE-0840501), and the North Carolina Biotechnology Center (NCBC 2009-IDG-1015).

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Figure 1.

ENDOR spectra at g_2 ($g = 2.018$) of the reaction intermediate “X” prepared using *E. coli* IspG and deuterated MEcPP/HMBPP-epoxides. (a) Mims ENDOR spectrum of “X” prepared using uniformly deuterated MEcPP; (b) Mims ENDOR spectrum of “X” prepared using **10**; (c) Mims ENDOR spectrum of “X” prepared using **11**; (d) Mims ENDOR spectrum of “X” prepared using **12**; (e) Mims ENDOR spectrum of “X” prepared using **13**; (f) the sum of (b)-(e); (g) Davies ^1H ENDOR spectrum of “X” prepared using **10** (solid line), showing the disappearance of the $a_{\text{iso}} = 12$ MHz ^1H signal (dashed lines), indicated by arrows. Mims ENDOR spectra shown in (a) - (e) are the sums of spectra taken at 30 different τ -values (from 132 ns to 1060 ns, in 32 ns steps), and are normalized according to their ^{31}P signal intensity. The percentages of ^2H enrichment were also taken into account when adding (b)-(e). Microwave frequency: 9.76 GHz. Magnetic field: 345.4 mT. $T = 20.0$ K.

Figure 2.

Q-band field-dependent Mims ENDOR spectra and simulations of “X” prepared using **10**. Black lines: experimental data; red lines: simulations. Microwave frequency: 34.05 GHz; $\tau = 740$ ns; $T = 20$ K. Simulation parameters: $A(^2H_a) = [1.8, 1.6, 1.8]$ MHz; $A(^2H_b) = [0.2, 0.0, 0.4]$ MHz; $A(^2H_c) = [0.5, 0.1, 1.1]$ MHz; $e^2qQ/h = 168$ kHz (2H_a) and 160 kHz (2H_b and 2H_c).

Figure 3.

HYSCORE spectra at g_2 ($g = 2.018$) of the reaction intermediate “X” using *E. coli* IspG and ^{13}C -labeled MEcPP. (a) $[\text{u-}^{13}\text{C}]$ MEcPP (14); (b) $[1,3,4\text{-}^{13}\text{C}_3]$ MEcPP (15); (c) $[2,3\text{-}^{13}\text{C}_2]$ MEcPP (16). The weaker ^{13}C signals in (c) are due to low enrichment.¹⁹ In (a) and (b), the diagonal peak at ~ 3.6 MHz is the superposition of ^{13}C signals having small (<1 MHz) hyperfine couplings from the labeled substrates and the double quantum transitions from protein ^{14}N , while in (c), this signal arise from double quantum transitions from protein ^{14}N . Microwave frequency: (a) 9.684 GHz; (b) 9.684 GHz; (c) 9.674 GHz. Magnetic field: (a) 342.3 mT; (b) 342.68 mT; (c) 342.5 mT. $\tau = 136$ ns. $T = 20.0$ K.

Scheme 1.

Reactions catalyzed by the proteins IspG (GcpE) and IspH (LytB).

Scheme 2.

Proposed structures for the reaction intermediate "X"

Scheme 3.

Isotopically labeled HMBPP-epoxides used in this study.

Scheme 4.

^{13}C -labeled MEcPPs used to generate “X”: * marks label positions