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Mechanistic Studies of the Protonation-Deprotonation Reactions for Type 1 and Type 2 Isopentenyl Diphosphate:Dimethylallyl Diphosphate Isomerase

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ABSTRACT

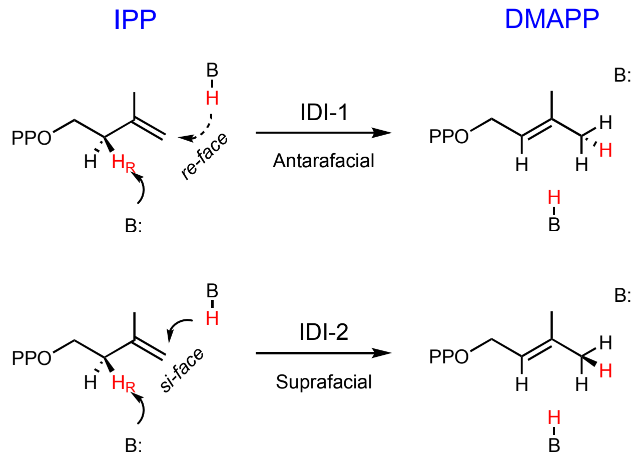
Type 1 and type 2 isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IDI-1 and IDI-2) catalyze the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the fundamental building blocks for biosynthesis of isoprenoid compounds. Previous studies indicate that both isoforms of IDI catalyze isomerization by a protonation-deprotonation mechanism. IDI-1 and IDI-2 are "sluggish" enzymes with turnover times of ~10 s^{-1} and ~1 s^{-1}, respectively. We measured incorporation of deuterium into IPP and DMAPP in D_2O buffer for IDI-1 and IDI-2 under conditions where newly synthesized DMAPP is immediately and irreversibly removed by coupling its release to condensation with L-tryptophan catalyzed by dimethylallyltrytophan (DMAT) synthase. During the course of the reactions, we detected formation of d_1, d_2, and d_3 isotopologues of IPP and DMAPP, which were formed during up to five isomerizations between IPP and DMAPP during each turnover. The patterns for deuterium incorporation into IPP show that d_2-IPP is formed in preference to d_1-IPP for both enzymes. Analysis of the patterns of deuterium incorporation are consistent with a mechanism involving addition and removal of protons by a concerted asynchronous process, where addition substantially precedes removal, or a step-wise process through a short-lived (< 3 ps) tertiary carbocationic intermediate. Work with mechanism-based inhibitors and related model studies by Richard and coworkers support a concerted asynchronous mechanism for the enzyme-catalyzed isomerizations.

Introduction

Isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IDI) catalyzes the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP),[1] the five carbon units required for biosynthesis of more than 70,000 isoprenoid natural products.[2] IPP and DMAPP are biosynthesized by the mevalonate (MVA) pathway in Eukaryotes, Archaea, and some Bacteria[3,4] or the methylerythritol phosphate (MEP) pathway in most Bacteria and plant chloroplasts.[5] IDI activity is required for synthesis of DMAPP from IPP in organisms that utilize the MVA pathway[6] and loss of IDI activity is lethal. In contrast, IPP and DMAPP are synthesized as a mixture from 4-hydroxy-DMAPP in organisms that utilize the MEP pathway. Although IDI activity is not essential for their survival,[7] most organisms that rely on the MEP pathway have IDI activity,[8] perhaps to balance the pools of IPP and DMAPP.

Two convergently evolved and structurally distinct forms of IDI are known.[9,10] IDI-1, discovered in the late 1950's, is present in some Bacteria and the cytoplasm of Eukaryotes.[8,11] IDI-2 is present in Archaea, other Bacteria, and plant chloroplasts.[12,13] Both isoforms catalyze the same reaction, although the organization of their active sites and catalytic residues responsible for isomerization are different. IDI-1 contains two divalent metal ions, typically Zn^{2+} and Mg^{2+} or Mn^{2+};[14,15] whereas, IDI-2 contains a single divalent metal, typically Mg^{2+}, and flavin mononucleotide (FMN), which is fully reduced in the active enzyme.[10]

The isomerizations catalyzed by IDI-1 and IDI-2 are stereoselective. Both enzymes bind IPP in an extended conformation where C4 in IPP becomes the *E*-methyl group in DMAPP. Both remove the *pro-R* hydrogen at C2 in IPP.[16,17] However, IDI-1 catalyzes an antarafacial isomerization of IPP to DMAPP by adding a hydrogen to the *re*-face of C4 in IPP, while IDI-2 catalyzes a suprafacial isomerization by adding a hydrogen to the *si*-face of C4 (Scheme 1).[16-20]

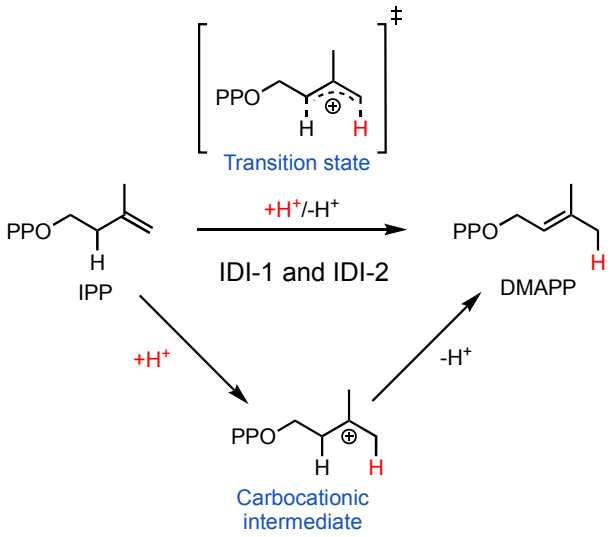


Scheme 1. Stereochemistry for isomerization of IPP to DMAPP by IDI-1 and IDI-2.

When incubations of IDI-1 or IDI-2 with IPP in D_2O are followed by ^{1}H-NMR, the reversible isomerization between IPP and DMAPP is accompanied by rapid incorporation of deuterium at the *E*-methyl group of DMAPP and at C4 and *pro-R* locus at C2 of IPP in accord with the stereochemistries reported for the two enzymes.[16-20] For IDI-1, all of the hydrogens, except those at C1, are eventually replaced by deuterium in both molecules upon prolonged incubation, reflecting the stereoselective, but not stereospecific, nature of the isomerization.[21] In contrast, only one of the hydrogens at C2 and the hydrogens at C4 are replaced during prolonged incubations with IDI-2, suggesting that it is substantially more stereoselective than IDI-1.[22] Careful analysis of the exchange reactions of IDI-1 in D_2O shows that two atoms of deuterium are incorporated into IPP during each turnover of the IDI-1·d_0-IPP complex.[23]

Several lines of evidence, including mechanism-based inhibition, site-directed mutagenesis, and studies with substrate analogues and transition state inhibitors, indicate that IDI-1 and IDI-2 catalyze isomerization via a [1.3] protonation-deprotonation mechanism.[24-35] Active-site acids and bases were identified from X-ray structures of IDI-1 complexed with an ammonium transition state/reactive intermediate analogue[25] and both enzymes with covalently attached mechanism-based irreversible inhibitors.[25,27,35] In the active site of IDI-1, a Zn^{2+}-Glu/Tyr motif protonates the C3-C4 double bond in IPP and a cysteine removes the proton at C2.[25] In contrast, a zwitterionic tautomer of reduced FMN ($FMNH_2$) is thought to be the acid-base catalyst for protonation of the double bond in IPP and abstraction of a proton from C2.[36]

The above work is consistent with either asynchronous concerted or stepwise mechanisms (Scheme 2), with a few noteworthy exceptions. Incubation of IDI-2 in D_2O with a vinyl thiomethyl analogue of IPP, where the methyl group was replaced with thiomethyl, resulted in rapid exchange of the C4 protons without isomerization of the double bond.[37] An IDI-1 mutant, where the nucleophilic cysteine that assists with proton removal was replaced by alanine, did not catalyze isomerization but was covalently inactivated by the epoxy analogue of IPP. [25] An X-ray structure of the adduct showed that the inhibitor alkylated the glutamate in the Zn^{2+}-Glu/Tyr protonation motif, suggesting protonation of the analogue by the glutamate was followed by alkylation of that residue by the protonated epoxide. Both observations are inconsistent with an asynchronous concerted protonation-deprotonation process. However, the carbocations generated by protonation of the thiomethyl and epoxy analogues of IPP are substantially more stable than the putative tertiary carbocation derived from IPP or DMAPP, and it is possible that they can be generated by the catalytic machinery in IDI, while the tertiary carbocation from IPP or DMAPP is not. This suggestion is consistent with the observation that the C67A mutant of IDI-1, which was inactivated by the epoxy analog of IPP, did not catalyze isomerization. It is also noteworthy that prolonged incubations of IDI-1 and IDI-2 with IPP/DMAPP do not result in alkylation of the active site.[21,22]

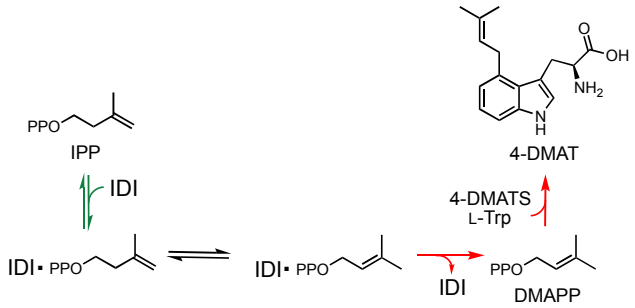


Scheme 2. Step-wise vs asynchronous concerted mechanisms for isomerization.

We now report experiments that examine the incorporation of deuterium into IPP and DMAPP for both IDI-1 and IDI-2 during incubations in D_2O buffer by mass spectrometry, which reveal formation of d_1, d_2, and d_3 isotopologues of IPP and DMAPP during each turnover. Analysis of the partitioning patterns of the isotopologues suggests that the protonation-deprotonation sequence is stepwise, involving a short-lived carbocationic intermediate ($\tau_{1/2} < 3$ ps), or a concerted asynchronous reaction, where protonation significantly precedes deprotonation.

Results

Trapping experiments. In order to detect multiple isomerizations between IPP and DMAPP during each turnover of IDI·d_0-IPP, we designed time-course experiments where DMAPP produced by isomerization of IPP by IDI is immediately and irreversibly consumed upon release by the dimethylallyltryptophan (4-DMAT) synthase catalyzed alkylation of tryptophan (Scheme 3). In a typical experiment, IPP was incubated with *E. coli* IDI-1 or *S. pneumoniae* IDI-2 and *Claviceps purpurea* 4-DMAT synthase in D_2O buffer where the [Trp] was 5-fold greater that the [IPP] and the specific activity of DMAT synthase was 40-fold higher than IDI-1 and 1000-fold higher than IDI-2. All of the buffer reagents, substrates, and proteins were thoroughly exchanged with deuterium by repeated lyophilization, and the reactions were initiated by the addition of IDI. As the reactions proceeded, samples were removed, and the enzymes were immediately separated from small molecules by ultrafiltration.



Scheme 3. Coupled reactions for removing DMAPP.

IPP and DMAT were separated on a reversed phase (RP) phenyl column (Figure 1). Fractions containing DMAT were lyophilized and analyzed by positive-ion mass spectrometry. Under these conditions, IPP eluted in the void volume. That fraction was lyophilized; the residue was dissolved in water; and IPP was purified by ion-exchange chromatography before analysis by negative ion electrospray mass spectrometry.[38] In control experiments run in H_2O, the intensities of ^{13}C and ^{18}O isotope peaks in unlabeled DMAT and IPP agreed with theoretical values, which are ~ 18% and 0.4% for DMAT and ~6% and 1.4% for IPP, respectively. There was no background at higher m/z values. In control experiments where IDI and DMAT synthase were incubated with DMAPP instead of IPP, no deuterium was incorporated into DMAT, demonstrating that DMAPP was efficiently trapped by DMAT synthase and did not isomerize to IPP, even under conditions where the initial [DMAPP] was substantially higher than the [DMAPP] achieved during isomerization of IPP (Figures S1 and S2).

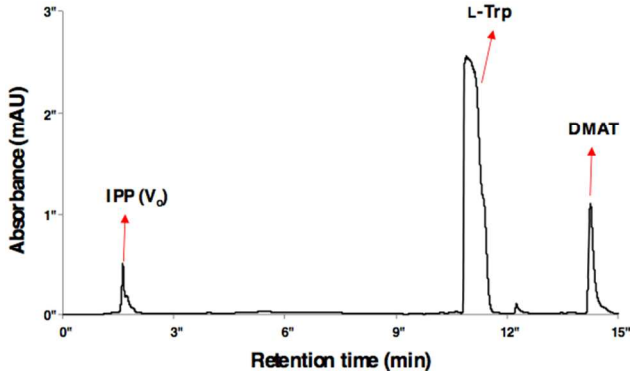


Figure 1. HPLC chromatogram of the assay mixture showing the retention times for IPP (void volume, V_o), L-Trp (~11 min) and DMAT (~14.5 min) upon elution with a 0-100% gradient of H_2O/acetonitrile and detection at λ_{210}.

Time courses for incorporation of IPP into DMAT, with immediate irreversible trapping of released DMAPP during isomerizations catalyzed by IDI-1 and IDI-2, are shown in Figure 2. Peaks for DMAT at m/z 296 (d_1-DMAT+Na^+), 297 (d_2-DMAT+Na^+), and 298 (d_3-DMAT+Na^+) revealed that up to three atoms of deuterium were incorporated into DMAPP during each turnover of IDI-1 (Figures S3A and S5-S12) and IDI-2 (Figures S3B and S12-S19). The mole fractions of d_1-, d_2-, and d_3-DMAT at each time point are given in Tables 1 and 2, respectively. These results show that newly released

DMAPP contained from one to three deuteriums (Tables 1 and 2), even at the earliest time points. It is noteworthy that the relative ratios for d_1-, d_2-, and d_3-DMAT observed early in the time courses, when most of the IPP in the sample did not contain deuterium, remained relatively constant up to ~25-40% conversion of IPP to DMAPP. At higher levels of consumption of IPP, the relative amounts of the d_2 and d_3 isotopologues increased modestly relative to d_1-DMAT as deuterium was incorporated into IPP (see below). Thus, the ratio of DMAPP isotopologues measured early in the reaction (up to ~25% conversion) reflects the incorporation of deuterium during each turnover.

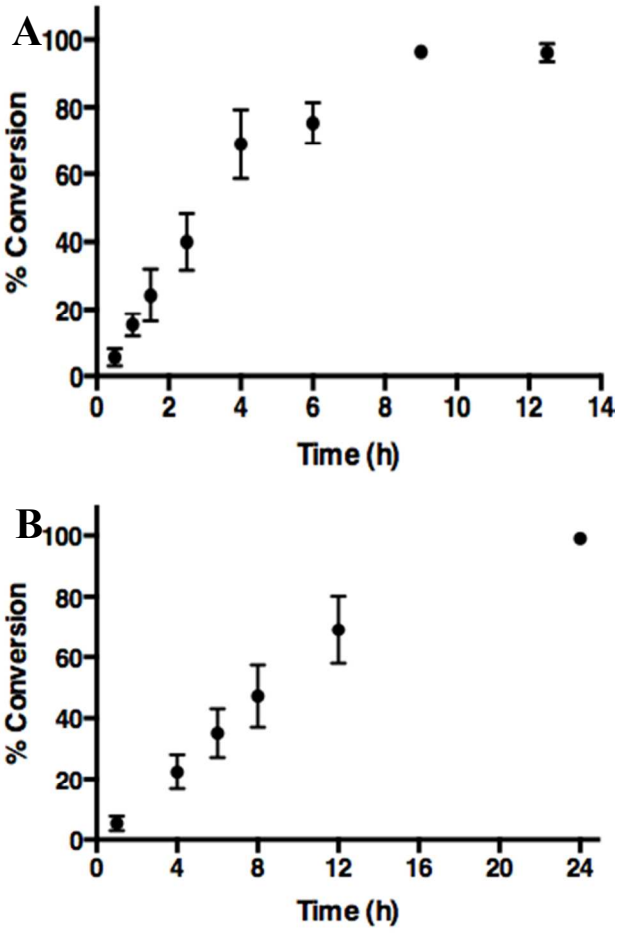


Figure 2. Percent conversion of IPP to DMAPP versus time for IDI-1 (part A, three independent assays) and IDI-2 (Part B, three independent assays). Average of three independent runs.

Table 1. Time-course for incorporation of deuterium into DMAT by IDI-1.[a,b]

Time (h)	Conversion (%)	DMAT+Na^+ [a,b] (Mole fraction)		
		m/z 296 d_1	m/z 297 d_2	m/z 298 d_3
0.5	6 ± 3	0.75 ± <0.01	0.21 ± <0.01	0.04 ± <0.01
1	15 ± 4	0.77 ± <0.01	0.20 ± <0.01	0.03 ± <0.01
1.5	24 ± 8	0.78 ± 0.02	0.19 ± <0.01	0.03 ± <0.01
2.5	40 ± 8	0.77 ± 0.01	0.20 ± < 0.01	0.03 ± <0.01
4	69 ± 10	0.74 ± 0.01	0.22 ± <0.01	0.04 ± <0.01
6	75 ± 6	0.71 ± 0.03	0.25 ± 0.02	0.04 ± <0.01
9	96 ± 1	0.66 ± <0.01	0.27 ± <0.01	0.07 ± <0.01

[a]2 mM IPP, 12 mM L-Trp, 0.1 mg/mL BSA, 10 mM $MgCl_2$, 1 μM IDI-1, 40 μM 4-DMATS pD 7.8, 30 °C, reaction volume 2.5 mL. [b]Average of three independent assays.

Table 2. Time-course for incorporation of deuterium into DMAT by IDI-2.[a,b]

Time (h)	Conversion (%)	DMAT+Na^+ [a,b] (Mole fraction)		
		m/z 296 d_1	m/z 297 d_2	m/z 298 d_3
1	5 ± 2	0.68 ± 0.02	0.26 ± 0.02	0.06 ± 0.02
4	22 ± 6	0.67 ± <0.01	0.26 ± <0.01	0.07 ± <0.01
6	35 ±8	0.65 ± <0.01	0.27 ± <0.01	0.08 ± <0.01
8	47 ± 10	0.63 ± <0.01	0.28 ± <0.01	0.09 ± <0.01
12	69 ± 11	0.59 ± <0.01	0.29 ± <0.01	0.12 ± <0.01
24	100	0.50 ± 0.03	0.31 ± <0.01	0.19 ± 0.03

[a]2 mM IPP, 10 mM L-Trp, 0.5 mM FMN, 5 mM $Na_2S_2O_4$, 0.1 mg/mL BSA, 10 mM $MgCl_2$, 10 nM *sp*IDI-2, 10 μM 4-DMATS pD 7.8, 30 °C, reaction volume 5.2 mL. [b]Average of three independent assays.

The LC-MS peak intensities for unlabeled IPP and its deuterium isotopologues were measured for the above time points for IDI-1 (Figures S4A and S20-27) and IDI-2 (Figures S4B and S28-S34). The mole fractions of d_1-, d_2-, d_3-IPP during the course of the reactions are given in Tables 3 and 4. Incorporation of deuterium into IPP at low conversions of IPP to DMAPP under conditions where free DMAPP is irreversibly trapped by DMAT synthase further supports the conclusion that interconversion of IPP and DMAPP within the enzyme-substrate/product complex is fast relative to their release. As seen for DMAPP, the distribution of IPP isotopologues during the first ~25% of the reaction reflects the pattern for incorporation of deuterium during each turnover of the enzymes.

Table 3. Time-course for incorporation of deuterium into IPP by IDI-1.[a,b]

Time (h)	Conversion (%)	IPP-H^+ (Mole fraction)			
		m/z 245 d_0	m/z 246 d_1	m/z 247 d_2	m/z 248 d_3
0.5	6 ± 2	0.93 ± 0.03	0.03 ± 0.02 (0.42)[c]	0.03 ± 0.01 (0.42)[c]	0.01 ± <0.01 (0.16)[c]
1	15 ± 4	0.92 ± 0.03	0.03 ± <0.01 (0.40)[c]	0.04 ± 0.03 (0.45)[c]	0.01 ± 0.01 (0.15)[c]
1.5	24 ± 8	0.88 ± 0.04	0.04 ± 0.01 (0.33)[c]	0.07 ± 0.01 (0.55)[c]	0.01 ± 0.01 (0.12)[c]
2.5	40 ± 8	0.80 ± 0.01	0.07 ± <0.01 (0.36)[c]	0.11 ± <0.01 (0.54)[c]	0.02 ± 0.01 (0.10)[c]
4	69 ± 10	0.66 ± 0.01	0.11 ± 0.01 (0.28)[c]	0.20 ± <0.01 (0.57)[c]	0.05 ± <0.01 (0.15)[c]
6	75 ± 6	0.41 ± <0.01	0.14 ± 0.01 (0.24)[c]	0.31 ± <0.01 (0.54)[c]	0.14 ± <0.01 (0.23)[c]
9	96	0.23	0.12 (0.16)[c]	0.4 (0.52)[c]	0.25 (0.32)[c]

[a]2 mM IPP, 12 mM L-Trp, 0.1 mg/mL BSA, 10 mM $MgCl_2$, 1 μM IDI-1, 40 μM 4-DMATS pD 7.8, 30 [o]C, reaction volume 2.5 mL. 9h is not average. [b]Average of two independent assays. [c]Normalized mole fractions for d_1-, d_2-, and d_3-IPP.

Table 4. Time-course for incorporation of deuterium into IPP by IDI-2.[a,b]

Time (h)	Conversion (%)	IPP-H^+ (Mole fraction)			
		m/z 245 d_0	m/z 246 d_1	m/z 247 d_2	m/z 248 d_3
1	5 ± 2	0.81 ± 0.09	0.02 ± 0.01 (0.11)[c]	0.17 ± 0.06 (0.85)[c]	0.01 ± <0.01 (0.04)[c]
4	22 ± 6	0.74 ± 0.03	0.04 ± 0.01 (0.16)[c]	0. 20 ± 0.02 (0.80)[c]	0.01 ± 0.01 (0.04)[c]
6	35 ± 8	0.67 ± 0.04	0.06 ± <0.01 (0.18)[c]	0.24 ± 0.02 (0.70)[c]	0.04 ± 0.01 (0.12)[c]
8	47 ± 10	0.53 ± 0.03	0.07 ± 0.02 (0.15)[c]	0.34 ± 0.01 (0.72)[c]	0.06 ± 0.01 (0.13)[c]
12	69 ± 11	0.15 ± 0.04	0.04 ± 0.01 (0.05)[c]	0.68 ± 0.01 (0.80)[c]	0.13 ± 0.03 (0.15)[c]

[a]2 mM IPP, 10 mM L-Trp, 0.5 mM FMN, 5 mM $Na_2S_2O_4$, 0.1 mg/mL BSA, 10 mM $MgCl_2$, 10 nM *sp*IDI-2, 10 μM 4-DMATS pD 7.8, 30 °C, reaction volume 5.2 mL. [b]Average of two independent assays. [c]Normalized mole fractions for d_1-, d_2-, and d_3-IPP.

The results for incorporation of deuterium into IPP during isomerizations catalyzed by IDI-1 and IDI-2 show that IDI-1 and IDI-2 catalyze up to five interconversions of IPP and DMAPP before releasing the substrate/product from the active site. While there is some scatter in the data for the isotopologues at early reaction times where the mole fraction of unlabeled IPP is high, the trend in the patterns for incorporation of deuterium is evident. Deuterium was incorporated into IPP early during the incubations with IDI-1 or IDI-2 and the amount of labeled IPP slowly increased as the reactions proceeded. The earliest samples from incubations of IDI-1 and IDI-2 with IPP contained a mixture of d_1-IPP, d_2-IPP, and d_3-IPP isotopologues where d_2-IPP was the predominant isotopologue for both enzymes. As the reactions progressed, the mole fraction of d_1-IPP decreased relative to d_2-IPP and d_3-

IPP (see amounts in parenthesis, Tables 3 and 4), the mole fraction of d_2-IPP did not change appreciably, and the mole fraction of d_3-IPP increased. The distribution of isotopologues clearly demonstrates that multiple protonation-deprotonation events occur between binding and release of IPP by the enzyme. The relative values of the mole fractions for the d_1, d_2, and d_3 isotopologues of IPP and DMAPP from incubations with IDI-1 and IDI-2 did not change substantially until more than ~25% of IPP was consumed. Afterward the mole fractions of d_2-IPP, and d_3-IPP isotopologues increased.

Analysis of deuterium incorporation. The deuterium labeling patterns for each turnover of IDI-1 and IDI-2 can be determined by extrapolation of the mole fractions of deuterium-containing isotopologues measured during the time course of the reaction back to t = 0. Our data show that the ratio of isotopologues did not change appreciably during the first ~25% of the reaction, where the amount of labeled IPP was low to relative to unlabeled IPP. We selected the mole fractions of IPP and DMAPP isotopologues determined at ~23% consumption of IPP for IDI-1 and IDI-2, respectively, as representative of the extrapolated values for our calculations. At this point in the time courses for IDI-1 and IDI-2, the reactions had proceeded to the point where the mole fractions of the isotopologues could be measured reliably, but the IPP pools still mostly consisted of d_0-IPP – 88% for IDI-1 and 74% for IDI-2.

Consider the distributions of deuterium isotopologues of IPP and DMAPP resulting from the first turnover of IDI-1, assuming that each isomerization reaction involves addition of a deuterium to the carbon-carbon double bond in IPP or DMAPP. At ~23% conversion of IPP to DMAPP, as measured by formation of DMAT, 12% of the remaining IPP contained deuterium, as measured by mass spectrometry. This corresponds to incorporation of label into 9% of the IPP [12% x (77/100)] present at the beginning of the reaction. Thus, at 23% conversion to DMAPP, 32% (9% + 23%) of the IPP present at t = 0 had been bound to IDI-1, deuterated, and released as IPP (28%) or DMAPP (72%). These percentages should closely approximate the values for each turnover. In a similar manner for IDI-2, 26% of the remaining IPP contained deuterium, which corresponds to incorporation of label into 20% of the IPP [26% x (77/100)] present at the beginning of the reaction. Thus, at 23% conversion to DMAPP,

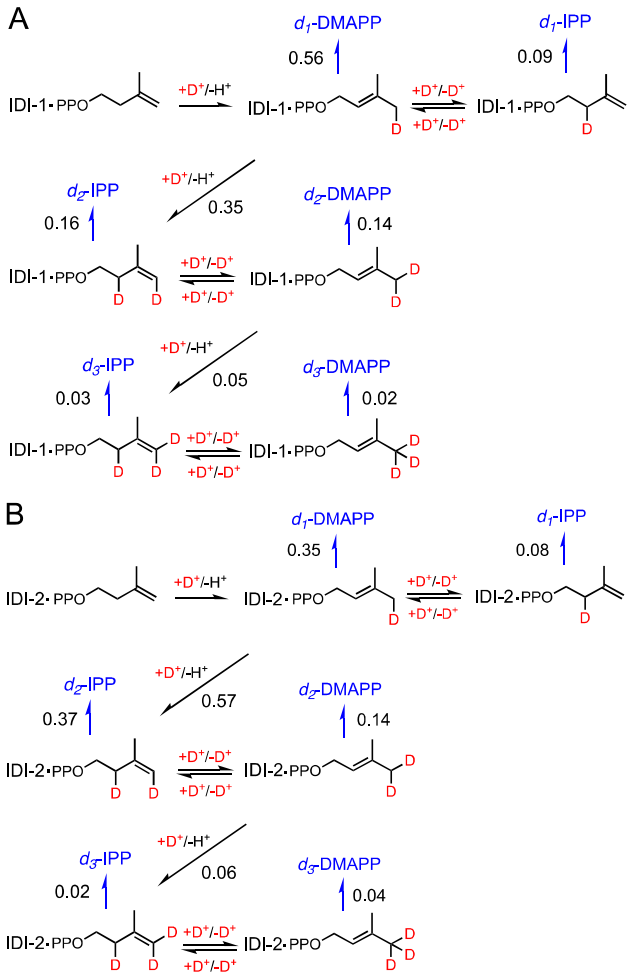
43% (20% + 23%) of the IPP present at t = 0 had been bound to IDI-2, deuterated, and released as IPP (47%) or DMAPP (53%). The mole fractional composition (χ) of the deuterated isotopologues produced during each turnover of IDI-1 and IDI-2, calculated from values for the distribution of d_1, d_2, and d_3 isotopologues of IPP and DMAPP in Tables 1-4. The respective percentages of deuterated IPP and DMAPP formed at ~23% conversion of IPP to DMAPP, are shown in Table 5.

Table 5. Mole fractions (χ) of IPP and DMAPP isotopologues formed during each turnover of d_0-IDI-1·IPP and d_0-IDI-2·IPP.

	χ_{IDI-1}	χ_{IDI-2}
d_1-IPP	0.09	0.08
d_2-IPP	0.16	0.37
d_3-IPP	0.03	0.02
d_1-DMAPP	0.56	0.35
d_2-DMAPP	0.14	0.14
d_3-DMAPP	0.02	0.04

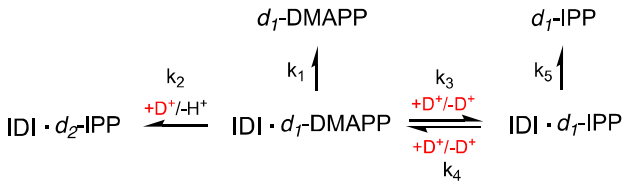
The simplest scenario for formation of the isotopologues of IPP and DMAPP during a turnover of IDI-1·d_0-IPP and IDI-2·d_0-IPP is the concerted mechanism presented in Scheme 4. Other possibilities are considered in the Discussion. While our experiments do not allow us to determine the relative rates for proton exchange with bulk solvent versus isomerization, the extensive incorporation of multiple deuteriums into IPP and DMAPP during each turnover demonstrates that a large fraction of the protons released during isomerization are not available for isomerization beyond the initial conversion of IPP to DMAPP. Thus, isomerization reactions where a deuteron is introduced and a proton is lost ($+D^+/-H^+$) are essentially irreversible. These steps include removal of a proton from C2 during the initial isomerization of d_0-IPP to d_1-DMAPP and removal of protons from the *E*-methyl group in DMAPP during subsequent DMAPP to IPP isomerizations. At 23% conversion of d_0-IPP, steps involving dissociation of the enzyme-ligand complexes are also essentially irreversible. The mole fractions of the

isotopologues in Table 5 correspond to the amount of each that is released from IDI during repeated isomerizations. The mole fraction of the original substrate (d_0-IPP) that passes through each irreversible step in the sequence of isomerizations can be calculated by subtracting the mole fractions of products released from IDI·IPP/DMAPP after the previous irreversible step from the mole fraction of IDI·IPP/DMAPP that passed through the previous irreversible step. Thus, for IDI-1·d_0-IPP → IDI-1·d_1-DMAPP, $\chi = 1.00 - 0 = 1.00$ for IDI-1·d_1-DMAPP; for IDI-1·d_1-DMAPP → IDI-1·d_2-IPP, $\chi = 1.00 - (0.56 + 0.09) = 0.35$ for IDI-1·d_2-IPP; and for IDI-1·d_2-DMAPP → IDI-1·d_3-IPP, $\chi = 0.35 - (0.16 + 0.14) = 0.05$ for IDI-1·d_3-IPP (Figure 4A). A similar analysis for IDI-2 gives the values shown in Figure 4B.



Scheme 4. Formation of IPP and DMAPP isotopologues during each turnover of d_0-IDI-1·IPP (part A) and IDI-2 (part B). Irreversible and reversible steps within the enzyme ligand complex are indicated by $+D^+/-H^+$ and $+D^+/-D^+$, respectively. Ligand release steps are indicated by blue arrows. Mole fractions (χ) are given for products formed irreversibly.

The formation of d_1, d_2, and d_3 isotopologues of IPP and DMAPP clearly demonstrates that rates for isomerization within and dissociation of the enzyme·IPP/DMAPP complexes are competitive. The mole fractions for IPP and DMAPP isotopologues shown in Scheme 4 reflect how newly bound IPP partitions during each turnover. As shown in Scheme 5, IDI·d_1-DMAPP partitions in three different directions, two of which – to IDI + d_1-DMAPP and to IDI·d_2-IPP – are irreversible. Thus, $\chi^{d_1\text{-DMAPP}}$ and $\chi^{\text{IDI}\cdot d_2\text{-IPP}}$ are directly related to k_1 and k_2, and $\chi^{d_1\text{-DMAPP}}/\chi^{\text{IDI}\cdot d_2\text{-IPP}} = k_1/k_2$. Since the E-methyl group of d_1-DMAPP only has two hydrogen atoms, the intrinsic value of k_2 for unlabeled DMAPP is roughly 50% greater than the corresponding rate constant for deprotonation of d_1-DMAPP. Furthermore, assuming that the deuteration of DMAPP does not significantly alter the rate of product dissociation (i.e., k_1) and the secondary isotope effect on k_2 is near unity, it follows that $k_1 \sim k_2$ for IDI-1·DMAPP and $k_1 \sim 0.4\ k_2$ for IDI-2·DMAPP. Thus, the rates for dissociation (k_1) and isomerization (k_2) are similar, and the lifetimes of the IDI·IPP/DMAPP complexes can be estimated from turnover times for *E. coli* IDI-1 ($\tau = 12$ s based on $k_{cat} = 0.08\ s^{-1}$) and *S. pneumonia* IDI-2 ($\tau = 1$ s based on $k_{cat} = 1\ s^{-1}$).

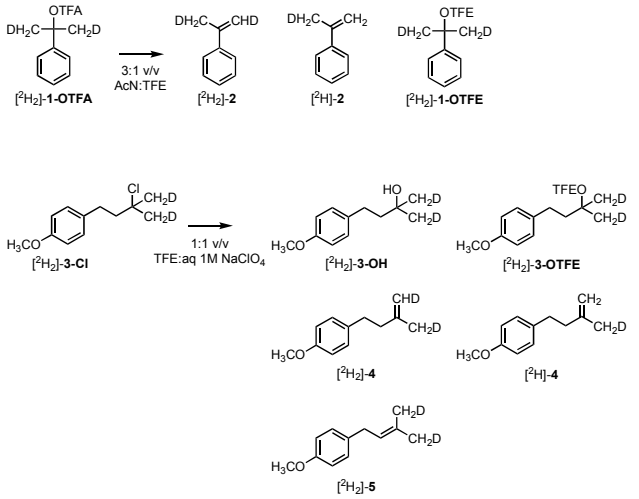


Scheme 5. Partitioning of IDI·d_1-DMAPP during each turnover.

IDI·d_1-DMAPP isomerizes to IDI·d_2-IPP (k_2) by loss of a proton and to IDI·d_1-IPP (k_3) by loss of a deuteron. The mole fraction ratios $\chi^{\text{IDI}\cdot d_2\text{-IPP}}/\chi^{d_1\text{-IPP}} = 3.9$ for IDI-1 and $\chi^{\text{IDI}\cdot d_2\text{-IPP}}/\chi^{d_1\text{-IPP}} = 7.1$ for IDI-2 strongly suggest a primary kinetic isotope effect (KIE), k_H/k_D, represented by relative rates for conversion of IDI·d_1-DMAPP to IDI·d_2-IPP (k_2) and IDI·d_1-IPP (k_3). Since removal of hydrogen is favored statistically over deuterium by a factor of two, $k_H/k_D = k_2/2k_3$. Unfortunately, the equilibrium

between IDI·d_1-DMAPP and IDI·d_1-IPP prevents us from determining unique relative values for k_1, k_2 and k_3. Relative values for rate constants shown in Scheme 5 were estimated from simulations using the mole fractions of d_1-DMAPP, d_1-IPP, and IDI·d_2-IPP in Schemes 4A and 4B along with different values for k_2/k_3 (Figures S62-63). None of the values for k_1–k_5 were unreasonably large or small within the range $2 < k_2/k_3 < 3.5$ for IDI-1 and $2 < k_2/k_3 < 4$ for IDI-2, but outside of these ranges one or more of the rate constants rapidly increased or decreased.

Primary deuterium kinetic isotope effect studies. We measured primary deuterium KIEs (k_H/k_D) for removal of a proton/deuteron from a -CDH_2 group in the elimination products produced during solvolysis of two extensively-studied systems, cumyl trifluoroacetate (**1**-OTFA) and 2-methyl-2-chloro-4-(4-methyoxyphenyl)butane (**3**-Cl) under solvolysis conditions reported by Richard and coworkers (Scheme 6).[39,40] The elimination products were separated from unreacted starting material and substitution products by chromatography. Fractions containing elimination products were combined, and the relative amounts of d_1 and d_2 isotopologues were determined by GC-MS. During preliminary experiments, we discovered that the relative intensities of mass spectral peaks characteristic of the isotopologues changed when the chromatographic peak was sampled at different stages of elution, suggesting the isotopologues partially resolved during chromatography. To address this issue, we collected ion currents in 0.1 m/z increments at 500 ms at intervals during the entire elution of the chromatographic peak and summed the ion currents for each m/z increment.



Scheme 6. Solvolysis of **1-OTFA** and **3-Cl**.

KIEs for the elimination reactions leading to **2** and **4** were determined from the relative intensities of peaks characteristic of their isotopologues. The molecular ion (M) for unlabeled **2** (m/z = 118) was accompanied by peaks at M-1 and M-3, which complicated analysis of peak intensities for the M and M+1 (^{13}C) peaks of the d_1 and d_2 isotopologues. However, the M-15 region (loss of a methyl group) was simpler with a strong peak at M-15 and a much smaller peak at M-1. This region in the spectrum for d_0- and d_1-α-methylstyrene (loss of CH_2D) had overlapping peaks at m/z 103, 104, and 105 for the C_8H_7 and C_8H_6D fragments and their ^{13}C isotopologues. Their relative intensities were determined from total ion current versus m/z plots by curve fitting as described in SI (Figure S35). A similar analysis was performed for the d_1 and d_2 isotopologues of olefin **4** based on their overlapping M and M+1peaks at m/z 177, 178, and 179 (Figure S36). Primary KIEs calculated from the relative intensities of peaks in the M-15 cluster for **2** and the M cluster for **4** were $k_{\mathbf{2}}^{H}/k_{\mathbf{2}}^{D} = 2.96 \pm 0.05$ and $k_{\mathbf{4}}^{H}/k_{\mathbf{4}}^{D} = 2.93 \pm 0.18$, respectively.

Discussion

IDI-1 and IDI-2 are convergently evolved enzymes that catalyze isomerization of IPP to DMAPP by a protonation-deprotonation reaction. A large body of work suggests that catalysis by both

enzymes proceeds by a step-wise mechanism through a tertiary carbocationic intermediate or by a concerted asynchronous mechanism through a late transition state with substantial carbocationic character.[18,19, 24, 27, 30,34,36,37]

The X-ray structures of IDI-1 complexed with a molecule of an amino analogue of IPP (NIPP), a transition state analogue for the carbocation (Figure 3),[25] and IDI-2 complexed with IPP (Figure 4)[30] are compatible with a protonation-deprotonation mechanism. In both enzymes, the ligands are bound in a pocket formed by highly conserved aromatic residues oriented to stabilize the formation of positive charge at C3 in the isopentenyl moiety through π-cation interactions. In IDI-1, an acidic glutamate (E116) and a basic cysteine (C67), are located on opposite faces of the isopentenyl group for protonation of *re*-face of the double bond and removal of the pro-*R* hydrogen at C2, consistent with the antarafacial stereochemistry for isomerization (see Scheme 1). In IDI-2, the zwitterionic tautomer of fully reduced FMN is both the acid and the base catalyst for the protonation/deprotonation steps.[30,36] The isopentenyl moiety in IPP is located in a plane ~3.5 Å above $FMNH_2$. The pro-*R* hydrogen at N5 in $FMNH_2$ forms a hydrogen bond with the backbone carbonyl group of M67 and the pro-*S* hydrogen is positioned to protonate the *si*-face of the C3-C4 double bond. The pro-*R* hydrogen at C2 in IPP is positioned to transfer to the flavin cofactor, consistent with the suprafacial stereochemistry seen for IDI-2. Q160, located on the opposite face of IPP, provides dipolar stabilization of developing positive charge in IPP.

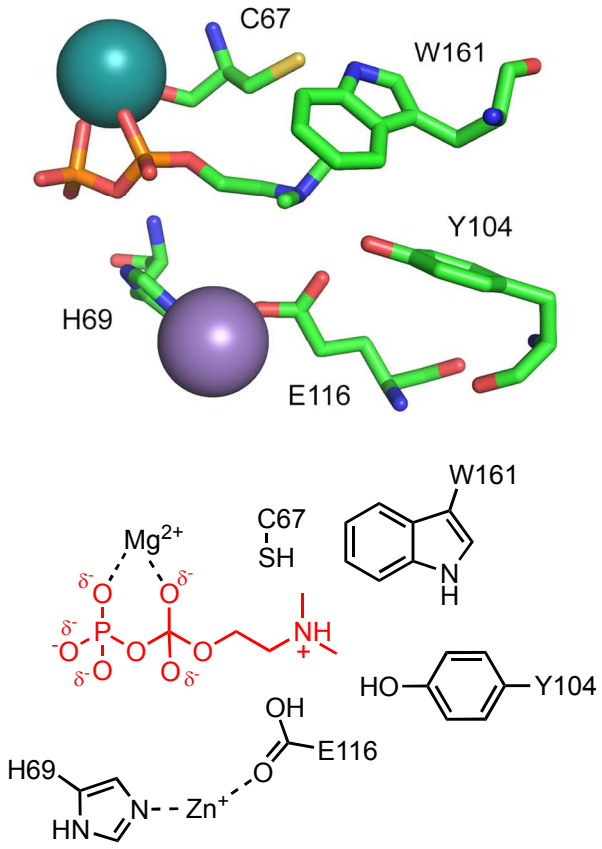


Figure 3. Active site of *Escherichia coli* IDI-1·NIPP.

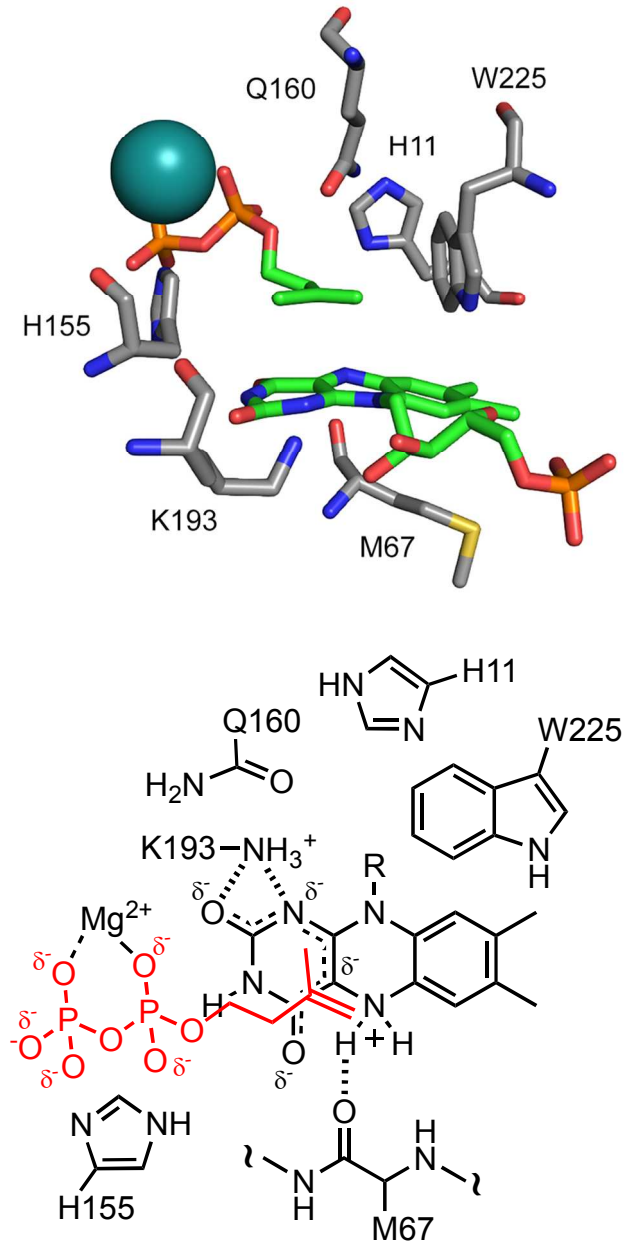
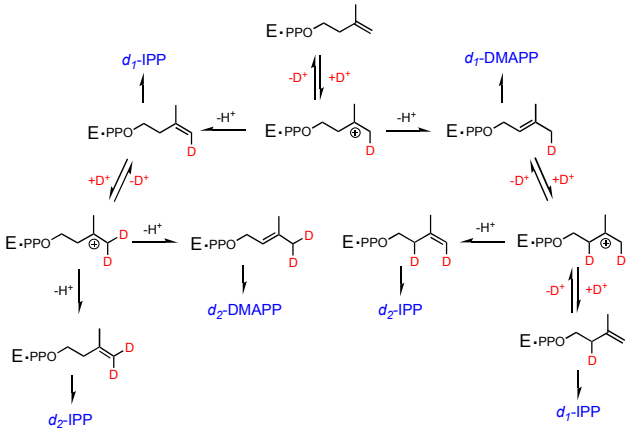


Figure 4. Active site of *Sulfolobus shibatae* IDI-2·IPP/DMAPP.

When the isomerizations catalyzed by IDI-1 and IDI-2 were run in D_2O buffer, one of the hydrogens at C2 and those at C4 in IPP and the *E*-methyl hydrogens in DMAPP were rapidly replaced by deuterium.[21,22] This pattern is consistent with the regio- and stereochemical preferences for the protonation-deprotonation reactions catalyzed by the enzymes. In related experiments, Richard and coworkers discovered that IDI-1 catalyzes at least two isomerizations - IPP to DMAPP and DMAPP back to IPP – during each turnover.[23] In the current study, we found that hydrogen atoms in IPP and

DMAPP were replaced sequentially by deuterium in up to five isomerization reactions during each turnover, which gave d_1, d_2, and d_3 isotopologues of both IPP and DMAPP. The lifetimes of IDI-1·IPP/DMAPP ($\tau = \sim 12$ s) and IDI-2·IPP/DMAPP ($\tau = \sim 1$ s) are relatively long, and the rates of product release and additional isomerization are similar for each isomerization within the IDI·IPP/DMAPP complexes.

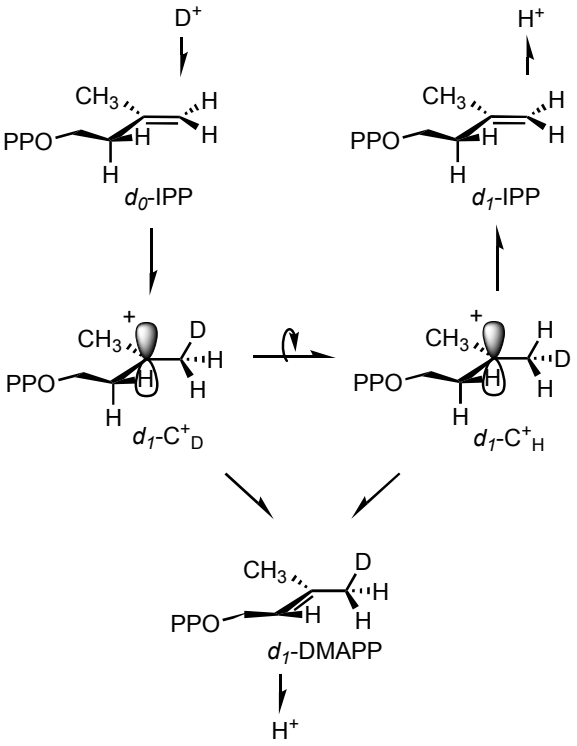
The reactions in Schemes 4A and 4B show the sequence of reactions for formation of deuterium isotopologues by a concerted asynchronous mechanism, where each time a deuterium is added to IPP or DMAPP, the compound is converted to its isomer. In this scenario d_1-DMAPP is formed during the first isomerization and is the immediate precursor for d_1-IPP and d_2-IPP. A more complex step-wise mechanism is shown in Scheme 7, where the d_1-carbocation generated from unlabeled IPP can give unlabeled IPP by removal of the deuterium at C4 or d_1-IPP or d_1-DMAPP by removal of a hydrogen from C2 or C4, respectively. Addition of another deuterium, to d_1-IPP or d_1-DMAPP is required to give d_2-IPP.



Scheme 7. Formation of d_1- and d_2-isotopologues by a step-wise mechanism.

In a concerted asynchronous mechanism, the only direct route to d_1-IPP and d_2-IPP is from d_1-DMAPP, where a primary KIE for removing a deuterium/hydrogen from C4 should favor formation of the d_2-isotopologue. In contrast, both d_1-IPP and d_1-DMAPP can be formed directly from the initial d_1-

carbocationic intermediate in a step-wise mechanism. d_2-IPP can only be formed by further isomerization of d_1-IPP or d_1-DMAPP. In this mechanism, one would anticipate that each turnover of unlabeled IPP would result in more d_1-IPP than d_2-IPP, in contrast to our observations. However, this result assumes that rotation of the deuteromethyl group in the d_1-carbocation is sufficiently fast relative to deprotonation to interconvert the d_1-C^+_D and d_1-C^+_H rotomers (Scheme 8). If the lifetime of d_1-C^+_D is substantially less than that for deuteromethyl rotation, formation of d_1-IPP does not compete with d_1-DMAPP and the predicted labeling patterns for formation of the IPP and DMAPP isotopologues in Schemes 4A and 7 become equivalent.



Scheme 8. Deuterium addition and hydrogen removal from conformational isomers of the d_1-carbocation.

Based on these considerations, our results are consistent with an asynchronous concerted mechanism or a step-wise mechanism, where the lifetime of the putative tertiary carbocation is less than that required for the *E*-methyl group to rotate by 120°. Although the barrier for rotation of a methyl group attached to the trigonal center in a tertiary carbocation is not known, one would expect it to be ~2-

3 kcal/mol based on the ~2-3 kcal/mol barrier for methyl rotation in 2-methylpropene,[42-45] corresponding to a lifetime (τ) of ~10^{-11} s for individual rotomers. These values are similar to the lifetimes for a variety of tertiary carbocations in aqueous solvents reported by Richard and coworkers, including the dimethylphenyl (cumyl) carbocation,[39] where $\tau \sim 10^{-10}$ s, represents carbocation-solvent combination, and a dimethylalkyl tertiary alkyl substituted carbocation,[40] where $\tau \sim 10^{-11}$ s represents rotation of a solvent molecule into a reactive conformation for combination. In both systems, the Richard group[40,45] found variations in the relative amounts of elimination and substitution products, which led them to conclude that substitution proceeded through a carbocationic intermediate, while elimination proceeded through a concerted process where the leaving group facilitated removal of the proton.

The primary hydrogen/deuterium KIEs we measured for removal of a methyl proton in the elimination reactions of **1**-OTFA and **3**-Cl, $k_H/k_D = 2.9$, were identical within experimental error. While we were not able to extract unique values for the k_2/k_3 ratio during isomerization of IDI·d_1-DMAPP to IDI·d_1-IPP and IDI·d_2-IPP (Scheme 5), the KIEs measured for solvolytic elimination fall in the middle of the range of reasonable simulated values and are consistent with similar concerted mechanisms for hydrogen removal in the solvolytic elimination and enzyme-catalyzed isomerization reactions.

A concerted asynchronous mechanism for the enzyme catalyzed isomerization of IPP to DMAPP that bypasses a highly electrophilic carbocationic intermediate has obvious advantages for preventing alkylation of nucleophilic residues in the active site. Both IDI isoforms catalyze highly regio- and stereoselective isomerizations. For IDI-2, only one of the hydrogens at C2 and both C4 hydrogens in IPP and all of the *E*-methyl hydrogens in DMAPP are exchanged, even upon prolonged incubation in D_2O. In contrast, IDI-1 is more promiscuous and all of the hydrogens in IPP and DMAPP, except those at C1 are exchanged. Thus, IDI-1 catalyzes proton exchange when the substrate is bound in different conformations that allow access of the active site glutamate and cysteine resides to hydrogens at C2, C4, and C5 in IPP and DMAPP without concomitant alkylation of active site nucleophiles or addition of

water. In contrast, epoxide and diene mechanism-based inhibitors, which are more readily protonated than carbon-carbon double bonds and are likely to generate protonated intermediates, readily alkylate C67 and E116. These observations are consistent with an asynchronous concerted mechanism for isomerization of the less reactive double bond in IPP and a stepwise protonation/alkylation mechanism for the epoxide and diene inhibitors. A concerted mechanism for IDI-2 requires that the zwitterionic form of $FMNH_2$ serves as both the acid and the base for protonation/deprotonation. The X-ray structure of IDI-2·IPP/DMAPP (Figure 4), shows protonated N5 and the carbonyl oxygen at C4 are positioned to facilitate a concerted [1.3] hydrogen shift through a concerted asynchronous six-membered transition state. Having both the acidic and basic functional groups located in each molecule may contribute to the enhanced stereoselectivity of IDI-2.

Conclusions

IDI-1 and IDI-2 are convergently evolved enzymes with different active site architectures. While turnover is rather sluggish, both enzymes catalyze up to five isomerization reactions each time IPP binds. Analysis of the patterns for incorporation of multiple deuterium atoms during each turnover reveals a marked preference formation of d_2-IPP over d_1-IPP, which is consistent with the kinetic isotope effects for removal of a hydrogen in related elimination reactions of tertiary alkyl derivatives. This behavior is also consistent with the extensive studies by Richard and coworkers indicating that solvolytic elimination reactions of tertiary alkyl derivatives follow concerted asynchronous pathways that are parallel to competing step-wise solvolytic substitution reactions. We conclude that IDI-1 and IDI-2 catalyze concerted asynchronous double bond isomerizations, which reduce the possibility of substrate inactivation by alkylation of their active site residues.

Associated Content

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: Synthesis and solvolysis experiments, including NMR spectra.. Measurement of kinetic isotope effects. Enzyme preparation and deuterium incorporation assays. Mass spectra for isotopologues and kinetic isotope effect determinations. Analysis of peak areas for kinetic isotope experiments. Simulations of time courses for partitioning of IDI·d_1-DMAPP.

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TOC

Mechanistic Studies of the Protonation-Deprotonation Reactions for Type 1 and Type 2 Isopentenyl Diphosphate:Dimethylallyl Diphosphate Isomerase

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