

Isopentenyl Diphosphate Isomerase Catalyzed Reactions in D₂O: Product Release Limits the Rate of This Sluggish Enzyme-Catalyzed Reaction

Venkatadurga Jonnalagadda, Krisztina Toth, and John P. Richard*

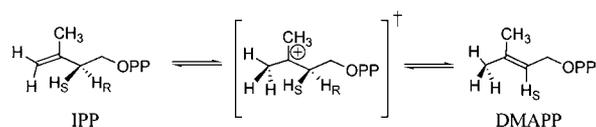
Department of Chemistry, University at Buffalo, Buffalo, New York 14260, United States

S Supporting Information

ABSTRACT: The *E. coli* isopentenyl diphosphate isomerase (IDI) catalyzed reaction of isopentenyl diphosphate (IPP) in D₂O gives a 66% yield of dimethylallyl diphosphate labeled with deuterium at the (*E*)-methyl group (*d*-DMAPP) and a 34% yield of IPP labeled with 1 mol of deuterium at C-2 (*d*-IPP). This shows that the release to D₂O of the initial product of the IDI-catalyzed reaction (*d*-DMAPP) is slower than its conversion to *d*-IPP. Product dissociation is therefore rate determining for isomerization of IPP with a rate constant $k_{\text{dis}} \approx k_{\text{cat}} = 0.08 \text{ s}^{-1}$. The data provide an estimated rate constant of $k_{\text{as}} = 6 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for binding of DMAPP to *E. coli* IDI that is similar to rate constants determined for the binding of N-protonated 2-amino ethyl diphosphate intermediate analogs to IDI from yeast [Reardon, J. E.; Abeles, R. H. *Biochemistry* **1986**, *25*, 5609–5616]. We propose that ligand binding to IDI is relatively slow because there is a significant kinetic barrier to reorganization of the initial encounter complex between enzyme, substrate, and an essential Mg²⁺ to form the Michaelis complex where the metal cation bridges the protein and the substrate diphosphate group.

Isopentenyl diphosphate isomerase (IDI) catalyzes 1,3-isomerization of isopentenyl diphosphate (IPP) to form dimethylallyl diphosphate (DMAPP)[†] through a tertiary carbocation-like transition state (Scheme 1).^{2,3} The central

Scheme 1



role for IPP and DMAPP in the biosynthesis of natural products,⁴ the deceptive simplicity of the catalyzed 1,3-proton transfer reaction, and the prominent role of tertiary carbocations in the development of our understanding of solvolysis and elimination reaction mechanisms⁵ have prompted penetrating mechanistic studies^{3,6–9} and X-ray crystallographic analyses of type 1 IDI.^{10–12} This work has identified several amino acid side chains essential for enzymatic activity and assigned their probable role in catalysis of the isomerization reaction.

We are interested in understanding the origin of the ~16 kcal/mol stabilization of the tertiary carbocation-like transition state at the active site of type 1 IDI.¹³ It has been shown that IDI catalyzes kinetically competent transfer of deuterium from solvent D₂O to the (*E*)-methyl group of the product DMAPP, and slow transfer of deuterium to the (*Z*)-methyl group of DMAPP.⁷ In the course of developing ¹H NMR assays of the IDI-catalyzed reaction in D₂O for use in studies of the reactions of truncated substrate analogs^{14,15} we have revisited and extended these isotope exchange experiments.

Type 1 *E. coli* IDI was purified from *E. coli* strain JM101 that contained the gene for IDI on a pARC306N expression vector. This strain, a generous gift from Prof. Dale Poulter, has been described earlier,^{16,17} except that the present construct contains an additional His-tag to facilitate protein purification. The protein was expressed according to published procedures^{6,18} and purified over a Ni-Chelating Sepharose Fast Flow from Pharmacia Biotech. Enzyme-catalyzed isomerization of IPP was assayed by monitoring the appearance of the signal for the *Z*-methyl hydrogen of DMAPP by ¹H NMR [see Supporting Information]. The procedures for determining a value of $k_{\text{cat}} = 0.08 \text{ s}^{-1}$ for turnover of IPP are also given as Supporting Information.

¹H NMR analyses [see Supporting Information] of the products of IDI-catalyzed reactions of IPP in D₂O showed at early reaction times a group of poorly resolved signals shifted upfield from the triplet for the 2-CH₂- hydrogen of IPP. Homonuclear decoupling of the signals for the 1-CH₂- and 2-CH₂- hydrogen of IPP was accomplished by irradiation at 3.96 ppm, the resonance frequency for 1-CH₂-. Decoupling causes the triplet for the 2-CH₂- hydrogen to collapse to a singlet at 2.295 ppm, and a simplification of the upfield signals, which now appear as a broad singlet at 2.272 ppm. Figure 1 shows partial ¹H NMR spectra obtained during the reaction of 21 mM IPP catalyzed by 3.5 μM *E. coli* IDI at pD 7.4 (100 mM phosphate buffer and 0.20 M KCl). The α-D at -CHD- groups of glycine and its derivatives perturbs the chemical shift of the remaining -H by ca. 0.02 ppm upfield from the signal for the parent -CH₂- hydrogen.^{19,20} We therefore assign the signal at 2.272 ppm to the 2-CHD- of 1. The apparent triplet [the coupling constants of 6.5 Hz for the splitting by the C-2 hydrogen and α-phosphorus are similar] for the C-1 methylene hydrogen of DMAPP appears at 4.35 ppm.

Received: March 4, 2012

Published: April 3, 2012

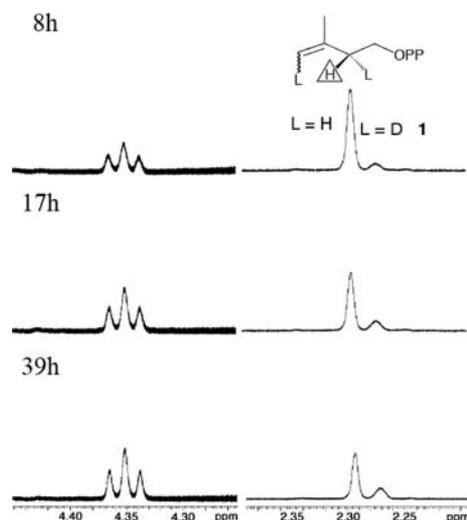


Figure 1. Partial ^1H NMR spectra of the products of IDI-catalyzed isomerization of IPP in D_2O , with homonuclear decoupling of the signals for the 1- CH_2 - and 2- CH_2 - of IPP by irradiation at the resonance frequency for 1- CH_2 - hydrogen. The triplet at 4.35 ppm and the apparent singlets at 2.295 and 2.272 are the signals for the C-1 hydrogen of DMAPP and the C-2 hydrogen of IPP and **1**, respectively. The experimental details for these ^1H NMR analyses are given in the Supporting Information.

Figure 2A shows the time course for isomerization of 26.5 mM IPP catalyzed by 28 μM *E. coli* IDI at pD 7.4 (20 mM

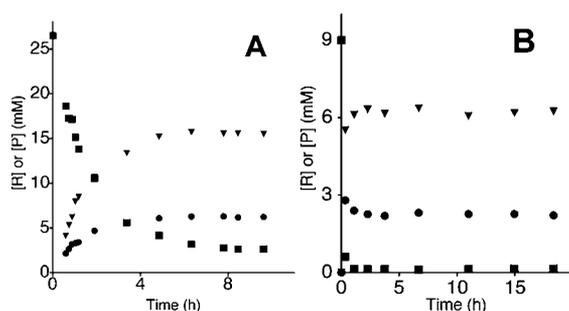


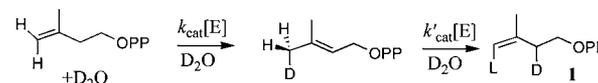
Figure 2. Time courses for the IDI-catalyzed reactions of IPP (\blacksquare) to form DMAPP_T (\blacktriangledown) and **1** (\bullet). DMAPP formed at early reaction times contains one atom of $-\text{D}$ (*d*-DMAPP, Scheme 3), but we observe the incorporation of multiple atoms of $-\text{D}$ as DMAPP and IPP achieve chemical equilibrium.⁷ (A) The isomerization of 26.5 mM IPP catalyzed by 28 μM *E. coli* IDI at pD 7.4 (20 mM imidazole), 400 mM NaCl, 10 mM MgCl_2 , and 0.5 mM DTT. (B) The isomerization of 9 mM IPP catalyzed by 71 μM *E. coli* IDI at pD 7.4 (20 mM imidazole), 400 mM NaCl, 10 mM MgCl_2 , and 0.5 mM DTT. The procedures for these experiments are given in the Supporting Information.

imidazole), 400 mM NaCl, 10 mM MgCl_2 , and 0.5 mM DTT. The decrease in $[\text{IPP}]$ (\blacksquare) was determined by monitoring the peak at 2.295 ppm for the 2- CH_2 - hydrogen, and the increase in $[\text{DMAPP}]_T$ (\blacktriangledown) and in $[\mathbf{1}]$ (\bullet) were determined by monitoring the triplet at 4.35 for the C-1 methylene hydrogen of DMAPP and the apparent singlet at 2.272 for the 2- CHD -hydrogen, respectively (Figure 1). Figure 2B shows the time course for the disappearance of IPP and the formation of **1** and DMAPP for isomerization of 9 mM IPP catalyzed by 71 μM *E. coli* IDI at pD 7.4 (20 mM imidazole), 400 mM NaCl, 10 mM

MgCl_2 , and 0.5 mM DTT. In this experiment the IPP is nearly completely consumed during the first 20 min of the reaction.

The data in Figure 2A and 2B are inconsistent with the sequential reaction shown in Scheme 2, where *d*-DMAPP is

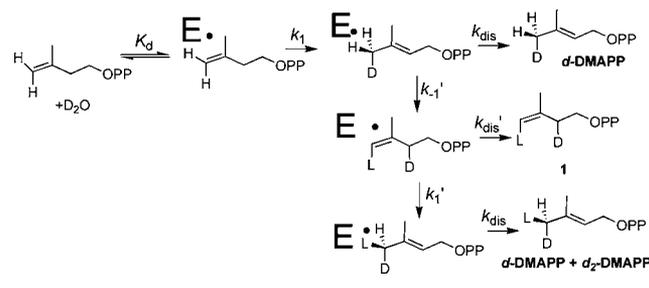
Scheme 2



released to D_2O and then converted to **1** in a second independent reaction. The requirement for product release will result in a lag in the formation of **1** as *d*-DMAPP accumulates, but this lag is not observed (Figure 2A). Instead, the product ratio $[\mathbf{1}]_T/[\text{DMAPP}]_T = 0.50$ is constant over the first three time points (0.9 h) and decreases to $K_{\text{eq}} = [\text{IPP}]_T/[\text{DMAPP}]_T = 0.36$ as total DMAPP and IPP achieve chemical equilibrium. This equilibrium constant is in fair agreement with the value of $K_{\text{eq}} = 0.45$ determined in earlier work.⁷ We also observe at later reaction times the extensive washout of $-\text{H}$ from the $-\text{CH}_3$ groups of DMAPP and IPP reported in the same earlier study.⁷

Figure 2A shows that *d*-DMAPP and **1** form at the same reaction velocity during the first 0.9 h of the reaction. This requires that the initial enzyme-catalyzed reaction of IPP gives *d*-DMAPP and **1** as products, so that the release of *d*-DMAPP to D_2O is slower than its conversion to **1** ($k_{-1}' > k_{\text{dis}}'$, Scheme 3). The initial product ratio $[\mathbf{1}]_T/[\text{DMAPP}]_T = 0.50$ is

Scheme 3



determined by the relative velocity for formation of *d*-DMAPP by k_1 , k_{dis} and for formation of **1** by k_1 , k_{-1}' , k_{dis}' (Scheme 3). At longer reaction times the signals for a poorly resolved broad pentet for the (*Z*)- CHD_2 group of d_2 -DMAPP were detected. We are uncertain of the yield (if any) for the formation of this product at early reaction times by k_1 , k_{-1}' , k_1' , k_{dis} (Scheme 3).

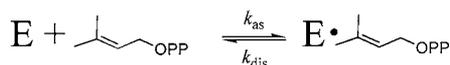
The initial product ratio $[\mathbf{1}]_T/[\text{DMAPP}]_T = 0.50$ from Figure 2B is the same as that determined for Figure 2A. The very high enzyme concentration used in this experiment results in the rapid consumption of IPP. We were surprised to observe (Figure 2B) that the concentration of **1** at early reaction times is larger than the concentration of **1** once chemical equilibrium with DMAPP is established! The IDI-catalyzed conversion of IPP to **1** is therefore *faster* than the formation of an equilibrium mixture of $[\text{IPP}]_T$ and $[\text{DMAPP}]_T$, because of the rapid washout of the C-2 hydrogen from IPP. This result is inconsistent with the sequential reaction shown in Scheme 2. It may be rationalized by the fast enzyme-catalyzed conversion of enzyme bound IPP to **1** through $\text{E} \cdot d\text{-DMAPP}$ (Scheme 3), provided the concentration ratio $[\text{E} \cdot \mathbf{1}]/[\text{E} \cdot d\text{-DMAPP}]$ during steady state turnover is larger than the ratio of the

concentration of the free species at chemical equilibrium in D_2O .

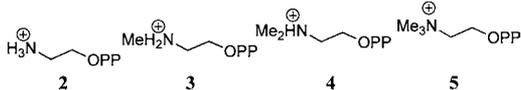
We conclude that conversion of $E \bullet d$ -DMAPP to **1** is faster than the release of d -DMAPP to D_2O ($k_{-1}' > k_{dis}$, Scheme 3). If product release is slower than the chemical steps at the enzyme active site, then it is rate determining for the turnover of IPP, so that $k_{dis} \approx k_{cat} = 0.08 \text{ s}^{-1}$ at 25°C . It is not unusual for product release to limit the rate for enzyme turnover. For example, the release of glyceraldehyde 3-phosphate is rate determining for TIM-catalyzed isomerization of dihydroxyacetone phosphate.²¹ However, product release from TIM is fast (*ca* 4000 s^{-1}) and rate determining only because the chemical isomerization of bound substrate is even faster.²¹ By contrast, *E. coli* IDI is a sluggish enzyme with $k_{cat} = 0.08 \text{ s}^{-1}$, and the enzyme–product complex has a surprisingly long lifetime of *ca.* 10 s.

The value of $K_d = 14 \mu\text{M}$ for inhibition of IDI-catalyzed isomerization of IPP by the product DMAPP¹⁷ may be combined with $k_{dis} \approx k_{cat} \approx 0.08 \text{ s}^{-1}$ for release of DMAPP to give $k_{as} \approx 6 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for ligand binding (eq 1 in Scheme 4). By comparison, the second-order rate constants k_{as} for

Scheme 4



$$K_d = k_{dis} / k_{as} \quad (1)$$



association of tight-binding inhibitors **2–5** (K_i is in the nanomolar to picomolar range) to IDI from yeast range from $10^5 \text{ M}^{-1} \text{ s}^{-1}$ for **2** to $10^3 \text{ M}^{-1} \text{ s}^{-1}$ for **5**.² We conclude that this slow ligand binding is a property of both the substrate and tight-binding cationic inhibitors. The high-affinity inhibitors are released exceedingly slowly to water.^{2,3} For example, the value of $k_{dis} < 5 \times 10^{-7} \text{ s}^{-1}$ for dissociation of **4** corresponds to a >16 day half-time for breakdown of the protein–inhibitor complex.²

There is likely a single mechanism-based explanation for the slow binding of *both* reactant DMAPP and inhibitors **2–5** to IDI. IDI requires two metal dications for activity and accepts a variety of dications at each of the two metal ion binding sites.⁶ One metal ion forms a distorted octahedral complex at a binding site that is partly composed of N-terminal amino acid side chain ligands that are disordered at the metal-free enzyme.¹² The second metal cation bridges the coordinated diphosphate group of the substrate with the IDI main-chain amide carbonyl oxygen of Cys-67 and the carboxylate side chain of Glu-87.¹² The slow binding of DMAPP and **2–5** to IDI shows that most encounters between the ligand and IDI are nonproductive. We suggest that the slow ligand binding reflects the substantial kinetic barrier to reorganization of the initial ligand• Mg^{2+} •enzyme encounter complex. This might include the barrier to reorganization of the side chains of Cys-67 and Glu-87 so that they are positioned to coordinate to the metal dication.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures for the synthesis of IPP, for the determination of k_{cat} for IDI-catalyzed isomerization of IPP using an ^1H NMR assay, and for ^1H NMR analyses of the yields of the products of IDI-catalyzed isomerization of IPP in D_2O . This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

jrichard@buffalo.edu

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was generously supported by Grant GM39754 from the National Institutes of Health. We thank Prof. Dale Poulter for providing us with the *E. coli* strain JM101 that contained the gene for IDI on an expression vector.

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