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Stereochemical Studies of the Type II Isopentenyl Diphosphate:Dimethylallyl Diphosphate Isomerase Implicate the FMN Coenzyme in Substrate Protonation

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The interconversion of isopentenyl diphosphate (IPP, **1**) and dimethylallyl diphosphate (DMAPP, **2**) by isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IDI) is a key reaction in the synthesis of the building blocks for isoprenoid compounds.^[1] Two structurally unrelated types of IDI have been identified. The type I enzyme (IDI-1) is a zinc metalloprotein that also requires Mg²⁺ for activity.^[2] It employs appropriate active site amino acid residues as general acid and base catalysts to carry out the isomerization reaction.^[2g, 3] In contrast, the type II IDI (IDI-2), discovered in 2001,^[4] is a flavoprotein that requires a reduced flavin mononucleotide (FMN_{red}, **3**) coenzyme in addition to Mg²⁺ for activity.^[5] The reaction catalyzed by IDI-2 is unusual in that it utilizes the redox active FMN coenzyme to perform a reaction that does not involve a change in the redox state of the substrate/product. This observation raises questions as to the exact role of the flavin coenzyme in the catalytic mechanism. In addition, several human pathogens, such as *Staphylococcus aureus*, rely exclusively on IDI-2 for the initiation of long-chain isoprenoid biosynthesis, whereas humans employ the structurally unrelated IDI-1. Thus, IDI-2 is a potential target for new antimicrobial agents.

Several recent studies provide evidence that IDI-2 may employ the reduced FMN coenzyme as an acid/base catalyst to mediate an allylic-1,3-proton addition/elimination reaction at C2 of IPP (**1**) (Scheme 1). Most notably, X-ray crystallographic studies of the reduced, substrate-bound form of IDI-2 from *Sulfolobus shibatae* revealed close proximity (2.9 Å) between N5 and the C2 atom of IPP, and the absence of obvious acidic/basic residues in the vicinity of the bound substrate.^[6] A substantial accumulation of positive charge on the reduced FMN during turnover as indicated by the kinetic linear free energy relationship (LFER) studies is also consistent with the role of N5 as a putative general acid/base catalyst.^[7] The latter observation is likely due to the transfer of the *pro-R* C2-H of IPP to the N5 atom of the reduced

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FMN coenzyme (**3** → **4** or **5** → **4**), which is at least partially rate-limiting in steady state IDI-2 turnover based on substrate deuterium kinetic isotope effect (KIE) studies.^[8]

Although the current data support a role for the reduced flavin in the protonation/deprotonation at C2 of IPP/DMAPP during turnover, the identity of the general acid/base catalyst responsible for mediating proton transfer at C4 remains elusive. Early studies by Poulter and co-workers^[9] showed that extended incubation in D₂O led to selective incorporation of deuterium into the C4 vinyl group of IPP and the (*E*)-methyl group of DMAPP. The strict C4 regiochemistry of solvent deuterium incorporation by IDI-2 strongly suggests a single IPP binding mode in the active site and the presence of a single acid/base catalyst that carries out C4 proton exchange. As depicted in Scheme 1, three scenarios can be envisioned. Since the N1 atom of the reduced flavin is proximal to the C4 position of IPP/DMAPP (3.9 Å, see Figure 1), it may function as the acid/base catalyst to mediate the proton transfer at C4 (Scheme 1A).^[6] This contention is supported by the fact that the p*K*_a of N1 of the reduced FMN is ~6.7 in solution^[10] and can be further modulated by a conserved lysine residue that is within hydrogen bonding distance (Lys193 in *S. shibatae* IDI-2, not shown). In addition, an intermediate accumulates during the pre-steady state of the IDI-2-catalyzed reaction that has a UV-visible absorption spectrum consistent with a neutral reduced flavin^[7, 11] However, the N1 proton of FMNH₂ (**5**) is expected to reside within the plane of the isoalloxazine ring and may not be optimally oriented for efficient proton transfer to C4 of the substrate. Thus, considering that the distance between N5 of FMN and C4 of IPP is only 3.6 Å (Figure 1), it is conceivable that N5 is the catalyst for both proton transfers at C2 and C4 of IPP/DMAPP (Scheme 1B).

Alternatively, proton transfer at C4 may be mediated by an amino acid residue as shown in Scheme 1C. A likely candidate is the conserved glutamine residue (Gln154 in *S. aureus* or Gln160 in *S. shibatae* IDI-2, Figure 1) which is optimally positioned (at a distance of 3.4–4.5 Å) to mediate proton transfer at the C4 position of IPP/DMAPP.^[6] While glutamine is not a typical acid/base catalyst, it may be part of a proton relay consisting of conserved amino acid residues (specifically His11 and Glu229 in *S. shibatae*, Figure 1). Indeed, site-directed mutagenesis of this glutamine residue led to a mutant enzyme with a substantially lowered *k*_{cat} value and an unperturbed *K*_m value for IPP.^[7] However, these studies do not exclude the possibility that Gln160 simply helps to position the substrate in the proper orientation for flavin-mediated proton transfer to occur. Given that Gln154 and the FMN coenzyme are on opposite faces of the bound substrate molecule (Figure 1), distinct stereochemical outcomes are expected for solvent deuterium incorporation at C4 of IPP for the mechanisms of Schemes 1A, B and 1C. Hence, chiral methyl analysis and proton inventory studies were conducted to probe the stereochemical course of the IDI-2 catalyzed isomerization reaction and examine the involvement of solvent-derived protons in catalysis. Accordingly, we synthesized both (*E*)- and (*Z*)-[4-³H]-IPP (**12** and **20**, Scheme 2) and individually incubated them with IDI-2 in D₂O buffer. A chiral methyl analysis assay^[12] was then used to determine the configuration of the (*E*)-4-methyl group of the corresponding DMAPP products.

The labeled substrates, (*E*)- and (*Z*)-[4-³H]-IPP, were synthesized by a reported procedure with slight modifications.^[13] The (*E*)- and (*Z*)-4-bromo-3-methyl-3-pentenol intermediates (**9** and **10**) were obtained in a combined yield of 30% (*E*:*Z* ~ 4:1) following separation by silica gel column chromatography. Tritium incorporation was accomplished by the incubation of **9** and **10** with *n*- and *t*-BuLi in THF at –78 °C, followed by quenching with [³H]-H₂O (20 mCi, 0.2 mL). When D₂O was used instead of [³H]-H₂O, more than 70% deuterium incorporation was detected by NMR spectroscopy. Subsequent tosylation in dry pyridine afforded the corresponding tosylates in 56% isolated yield ((*E*)- and (*Z*)-**11**: 0.09 μCi/μmol and 0.15 μCi/μmol, respectively). Coupling with pyrophosphate was carried out

following a known procedure^[14] (51–77% yield, (*E*)- and (*Z*)-[4-³H]-IPP (**12** and **20**): 0.033 $\mu\text{Ci}/\mu\text{mol}$ and 0.068 $\mu\text{Ci}/\mu\text{mol}$, respectively).

The labeled substrates **12** and **20** thus obtained were incubated with IDI-2 from *S. aureus*^[11] under anaerobic conditions in the presence of FMN, Mg^{2+} and sodium dithionite.^[15] The reactions were performed under single-turnover conditions (2 mM IDI-2:FMN, 1.8 mM substrate) and were quenched after approximately 1.5 s with HCl/MeOH (3:1). This protocol was necessary to generate sufficient quantity of chiral product for down stream analysis, while preserving the chirality at the (*E*)-4-methyl group of DMAPP, which would otherwise be lost during an extended incubation due to the facile reversibility of the IDI-2 catalyzed reaction ($K_{\text{eq}} = 1.4$).^[16] Following the acid quench, the derivatized DMAPP products^[17] were extracted with petroleum ether and, after solvent evaporation, the isolated products were subjected to Kuhn-Roth oxidation.^[18] The nascent acetic acid samples (formed in radiochemical yields of 33–44%) were converted to their respective CoA thioesters (**14** and **21**) and their chiralities were determined using the malate synthase/fumarase chiral methyl assay.^[12] Authentic (*S*)- and (*R*)-[2-²H,³H]-acetate standards were also prepared^[19] and analyzed using the same chiral methyl assay for comparison with the IDI-2-derived acetate samples.

The results of the chiral methyl analysis for the authentic acetate standards and for the acetate samples derived from **12** and **20** after incubation with IDI-2 are listed in Table 1. *F* values of 74 and 25 were determined for the authentic (*R*)- and (*S*)-acetate standards, respectively, which are near the values expected for enantiomerically pure samples (*F* = 79 and 21, respectively).^[12] Likewise, *F* values of 67 and 32 (corresponding to 59% and 62% ee of the (*R*) and (*S*) configurations) were obtained for acetate derived from (*E*)- and (*Z*)-[4-³H]-labeled IPP (**12** and **20**), respectively.

Although the ee values of the acetate samples derived from **12** and **20** are modest, these data clearly indicate that (*E*)- and (*Z*)-[4-³H]-IPP are deuterated in the active site to yield DMAPP with chiral (*E*)-4-methyl groups of the (*R*) and (*S*) configuration, respectively. On the basis of current structural models of the IDI-active site in complex with FMN, IPP, and Mg^{2+} (Figure 1), these stereochemical data are inconsistent with a mechanism of IDI-catalysis involving substrate protonation by an active site acid (Scheme 1C). Instead, the data indicate that the proton is added to the double bond of IPP from the side of the molecule facing the reduced FMN coenzyme, thus supporting a role for N1 or N5 of flavin in the protonation of IPP at C4 (Scheme 1A, B and 3A, B). The *F* values determined for the DMAPP-derived acetate samples, which differ from those determined for the chiral acetate standards, likely indicate that the reverse reaction has occurred to a significant extent prior to the acid quench of the IDI-2 reaction. This would reduce the fraction of chiral acetate in the samples via exchange of the C4 proton of IPP/DMAPP with solvent deuterium, thereby perturbing the experimentally determined *F* values toward 50.

To gain additional insight into the mechanism of solvent proton incorporation by IDI-2, solvent kinetic isotope effect (SKIE) and proton inventory studies of the IDI-2-catalyzed reaction were performed.^[20] At pH 8.0, the SKIEs on the IDI-2-catalyzed reaction are $^{\text{D}_2\text{O}}k_{\text{cat}}/K_{\text{m}} = 3.8$ and $^{\text{D}_2\text{O}}k_{\text{cat}} = 1.4$, suggesting that bonds to solvent exchangeable proton(s) are likely breaking in the rate-limiting transition state(s) (Figure S2). Control experiments demonstrate that the apparent SKIEs are not due to an increase in solution viscosity caused by the substitution of H_2O with D_2O (Figure S4). The proton inventory curve of the IDI-2-catalyzed reaction under saturating IPP concentrations is shown in Figure S3 along with fits to various forms of the Gross-Butler equation.^[21] The curve is dome-shaped and most readily explained by a model involving the transfer of a single solvent-exchangeable proton with a fractionation factor $\phi = 0.3$ in the transition state of a step that is

only partially rate-limiting ($c_{\text{vf}} + c_{\text{r}} = 2.4$, where c_{vf} and c_{r} are forward and reverse commitments to catalysis). A fractionation factor of 0.3 corresponds to a normal SKIE of 3.3 and is consistent with bond cleavage to a solvent exchangeable proton and perhaps reflects the transfer of a proton to the IPP double bond (**5** $\rightarrow$ **4** or **4** $\rightarrow$ **3**, Scheme 1A, B).

In summary, chiral methyl analysis of the DMAPP products derived from the IDI-2 catalyzed reaction with (*E*)- and (*Z*)-[4-³H]-IPP (**12** and **20**) in D₂O provides strong support for a chemical mechanism involving a flavin-mediated protonation at the vinyl C4 position of the bound IPP substrate. In addition, SKIE and proton inventory studies suggest that this proton transfer may be partially rate-limiting to steady state IDI-2 turnover. When taken into consideration with the body of mechanistic and structural studies of IDI-2,^[6–8, 11, 22] these results are most consistent with a mechanism involving reduced flavin-mediated acid/base chemistry at both C2 and C4 of IPP/DMAPP (Scheme 1A, B). The relative feasibility of the two pathways in Scheme 1A, B depends largely on the identity of the reduced flavin species that accumulates in the pre-steady state of the IDI-2-catalyzed reaction. A comparison of the UV-visible absorption spectrum of IPP-bound IDI-2 with other reduced flavoenzymes^[7, 11] suggests that 1,5-dihydro-FMNH₂ (**5**) accumulates upon IPP binding, which would favor the mechanism shown in Scheme 1A. In this scenario, the protonation at C4 of IPP mediated by N1 of the reduced FMN (perhaps assisted by Lys186, Lys193 in *S. shibatae*) and the deprotonation at C2 by N5 may occur in either a stepwise or concerted fashion. However, as noted above, the N1 proton of **5** may not be in an optimal position for transfer to the double bond of IPP. Thus, the mechanism in Scheme 1B is currently favored, in which N5 catalyzes the sequential protonation (at C4) and deprotonation (at C2) of IPP. Clearly, the results presented herein have unambiguously established an unusual function of flavin coenzymes in biocatalysis. Interestingly, a similar role for reduced flavin has also been implicated in the mechanisms of several newly characterized enzymes.^[23]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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20. It should be noted that the activity of IDI-2 reconstituted with reduced 1-deaza-FMN is comparable to that of wild type enzyme.^[14] Since the C1 pKa of 1-deaza-FMN is ~ 5.8,^[1] this coenzyme is protonated predominantly at O2 and O4 to form FMNH₂ in free solution. However, the C1 anion may be a more important resonance contributor in the IDI-2 active site given the presence of Lys186 which is ~ 3 Å away.
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Figure 1.
Structural model of the *S. shibatae* IDI-2 active site (PDB entry 2ZRY) complexed with IPP (1).

Scheme 1.

Putative chemical mechanisms for IDI-2 involving the reduced FMN as an acid/base catalyst.

Scheme 2.

Synthesis of (E)- and (Z)-[4-³H]-IPP (**12** and **20**) and determination of the stereochemical course of the IDI-2 reaction by chiral methyl analysis, assuming that the solvent deuterium is added to C4 from the *si*-face of IPP during turnover.

Scheme 3.

The stereochemical course of the IDI-2 catalyzed reactions with **12** and **20** in D_2O , illustrated with mechanisms in which A) N1 and N5 of reduced FMN, B) N5 alone mediate acid/base chemistry at C4 and C2 of IPP.

Table 1

Chiral methyl analysis of the IDI-2-catalyzed reaction.

Substrate/Standard	<i>F</i> Value ^[a]	% ee ^[b]	Stereochemistry
(R)-acetate	74	83	(R)
(S)-acetate	25	86	(S)
12	67 ± 3	59 ± 10	(R)
20	32 ± 7	24 ± 24	(S)

^[a] *F* value = percentage of tritium retention in the fumarase reaction.^[b] % ee = [(|F-50|)/29]×100