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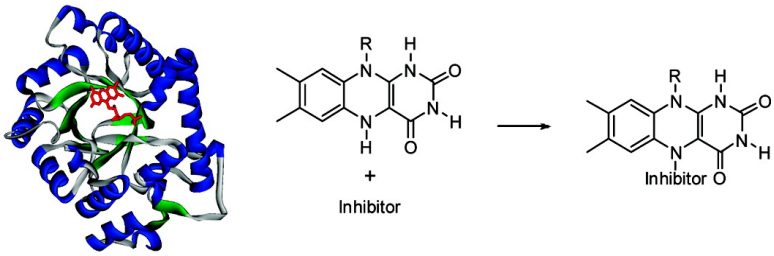

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Type II Isopentenyl Diphosphate Isomerase: Irreversible Inactivation by Covalent Modification of Flavin

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Abstract: Isopentenyl diphosphate isomerase (IDI) catalyzes the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the basic building blocks of isoprenoid molecules. Two structurally unrelated classes of IDI are known. Type I IPP isomerase (IDI-1) utilizes a divalent metal in a protonation−deprotonation reaction; whereas, the type II enzyme (IDI-2) requires reduced flavin. Epoxy, diene, and fluorinated substrate analogues, irreversible inhibitors of IDI-1, were analyzed as mechanistic probes for IDI-2. 3,4-Oxido-3-methyl-1-butyl diphosphate (eIPP), 3-methylene-4-penten-1-yl diphosphate (vIPP), and 3-(fluoromethyl)-3-buten-1-yl diphosphate (fmIPP) inactivate IDI-2 through formation of covalent adducts with the reduced flavin. UV−visible spectra of the inactivated complexes are consistent with modification of the isoalloxazine ring at position N5. vIPP and fmIPP are also alternate substrates with isomerization competing with alkylation of the flavin cofactor. (*Z*)-3-(Fluoromethyl)-2-buten-1-yl diphosphate ((*Z*)-fmDMAPP) and (*Z*)-3-(difluoromethyl)-2-buten-1-yl diphosphate ((*Z*)-dfmDMAPP) are alternate substrates, which are isomerized to the corresponding IPP derivatives. The rates of isomerization of fmIPP and (*Z*)-fmDMAPP are approximately 50-fold less than IPP and DMAPP, respectively. dfmIPP is not an irreversible inhibitor. These studies indicate that the irreversible inhibitors inactivate the reduced flavin required for catalysis by electrophilic alkylation and are consistent with a protonation−deprotonation mechanism for the isomerization catalyzed by IDI-2.

Introduction

The conversion of isopentenyl diphosphate (IPP) to dimethylallyl diphosphate (DMAPP), catalyzed by IPP isomerase (IDI), is an important step in the early stage of isoprenoid metabolism. DMAPP is the initial electrophilic substrate for the chain elongation reactions that lead to most of the isoprenoid compounds found in nature, including mono-, sesqui-, and diterpenes, carotenoids, sterols, ubiquinones, and dolichols.[1] For organisms that synthesize isoprenoid units by the mevalonate (MVA) pathway, IDI is an essential enzyme.[2] IDI is also found in many organisms that utilize the methylerythritol phosphate (MEP) pathway to produce both IPP and DMAPP, where the enzyme presumably serves to balance the pools of IPP and DMAPP.[3]

Two isoforms of IPP isomerase have been identified. Type I IPP isomerase (IDI-1) has been studied for over 40 years.[4−8] The enzyme requires two divalent metals−Mg^{2+} facilitates substrate binding through the diphosphate moieties in IPP and DMAPP and Zn^{2+} is required for catalysis.[4−9] In 2001, a second form of IDI was reported.[10] The structure of the "type II enzyme" (IDI-2) is unrelated to IDI-1, and the enzyme requires a divalent metal and reduced flavin mononucleotide (FMN) for activity.[10−12] There is no strict correlation between the two IDI isoforms and biosynthetic pathways to IPP.[13] Organisms that synthesize IPP and DMAPP from MVA have IDI-1 (eukaryotes) or IDI-2 (archaea and a few bacteria), while organisms that utilize the MEP pathway have IDI-1 (plant chloroplasts and bacteria) or IDI-2 (bacteria). The co-occurrence of IDI-2 and the MVA pathway in *Streptococcus pneumoniae* and *Staphylococcus aureus* represents an attractive target for antibacterial drug design.

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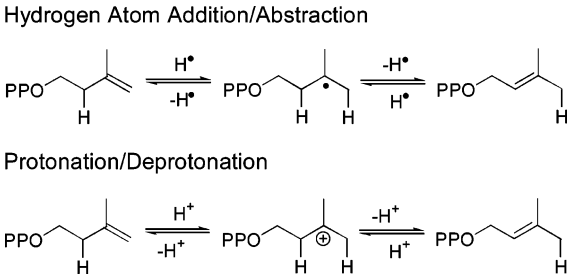
Scheme 1. Proposed Mechanisms for Isomerization of IPP and DMAPP by IDI-2



The mechanism for isomerization catalyzed by IDI-1 has been established by several lines of evidence. In particular, studies with IPP analogues have provided extensive support for substrate protonation to generate a transient carbocationic intermediate. Epoxide and diene analogues were shown to irreversibly inhibit the enzyme through formation of covalent adducts with active site cysteine and glutamate residues.[14,15] In both cases, protonation serves to activate the analogue for attack by an active site nucleophile. *N*,*N*-Dimethyl-2-amino-1-ethyl diphosphate (nIPP), a reactive intermediate analogue with a positively charged ammonium group, binds to the type I enzyme with subnanomolar affinity.[16] In addition, IPP and DMAPP analogues substituted with powerful electron-withdrawing fluorine groups are poor substrates for isomerization.[16,17] These studies provide evidence for a mechanism that involves protonation at the double bond of IPP, followed by deprotonation of the carbocationic intermediate to generate DMAPP as the product.

Although the reactions catalyzed by IDI-1 and IDI-2 are identical, the two enzymes utilize different cofactors. Of particular interest is the role of the flavin cofactor. Although flavins normally mediate redox reactions, there are examples where the cofactors fulfill other roles.[18] Several mechanisms have been suggested for IDI-2 and the associated role of reduced flavin.[18] A variety of studies with substrate and flavin analogues have been interpreted as evidence for either protonation-deprotonation or radical-based mechanisms (Scheme 1).[11,19] We now report experiments with IDI-2 and a series substrate analogues that inactivate the enzyme by electrophilic alkylation of the flavin cofactor.

Experimental Section

Materials. 3-Oxiranyl-3-buten-1-yl diphosphate (oIPP), 3-cyclopropyl-2-buten-1-yl diphosphate (cDMAPP), 3-cyclopropyl-3-buten-1-yl diphosphate (cIPP), (*Z*)-3-difluoromethyl-2-buten-1-yl diphosphate ((*Z*)-dfmDMAPP), (Z)-3-fluoromethyl-2-buten-1-yl diphosphate ((*Z*)-fmDMAPP), (*E*)-3-fluoromethyl-2-buten-1-yl diphosphate ((*E*)-fmDMAPP), 3-methylene-4-penten-1-yl diphosphate (vIPP) and 3-methyl-2,4-pentadienyl diphosphate (vDMAPP) were available from previous studies.[15,20−22] 3,4-Oxido-3-methyl-1-butyl diphosphate (eIPP) was provided by Sam Barkley. 3-Fluoromethyl-3-buten-1-yl diphosphate (fmIPP) was prepared as previously described.[23] IDI-2 from *Thermus thermophilus* was expressed in *Escherichia coli* and purified as described.[12,24] Glycerol, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and guanidinium·HCl were from USB Corporation. D_2O was purchased from Cambridge Isotopes. Other reagents, unless specified, were purchased from Sigma.

Inactivation Assays. Enzyme (250−500 nM) was preincubated with an analogue at 37 °C in a 200 μL solution of 200 mM HEPES (pH 7.0) containing, 40 μM flavin mononucleotide (FMN), 2 mM NADPH, and 2 mM $MgCl_2$. Portions of the mixture were removed periodically and diluted 5- or 10-fold into IPP assay buffer containing final concentrations of 200 mM HEPES (pH 7.0), 40 μM FMN, 2 mM NADPH, 2 mM $MgCl_2$, 50 μM [1-^{14}C]IPP (10−15 μCi/μmol). Isomerase assays were then performed as described previously.[12] Kinetic constants for inactivation were calculated as previously described.[15]

Competitive Inhibition Assays. Kinetic constants for reversible inhibition were determined as previously described[12] using 200 mM HEPES buffer, pH 7.0, containing 2 mM NADPH, 2 mM $MgCl_2$, 0.14 mg/mL BSA, at 37 °C for 2, 5, 10, 20 μM IPP and the following inhibitor concentrations: (*Z*)-fmDMAPP−0, 36, 72, 144 μM; cIPP−0, 75, 150, 300 μM; (*Z*)-dfmDMAPP−0, 375, 750, 1500 μM.

UV−Vis Assays. UV−visible absorbance spectra of the enzyme−analogue complexes were measured at 37 °C under anaerobic conditions, employing either photochemical or NADPH reduction methods as previously described.[12] Measurements of inactivated complexes were performed as follows: flavin-bound IDI-2 (50 μM) and analogue (100−400 μM) were incubated at 37 °C for 2 h in 400 mM HEPES buffer, pH 7.0, containing 2 mM $MgCl_2$, and 2−4 mM NADPH. The samples were washed three times under aerobic conditions with 10 mM NH_4HCO_3, pH 7.8, at 4 °C using a Microcon centrifugal filter (molecular weight cut off of 30 KDa). UV−visible spectra of samples diluted into 10 mM NH_4HCO_3, pH 7.8, were recorded on an Agilent 8453 diode array spectrophotometer. Samples were subsequently concentrated and diluted into 20 mM NaH_2PO_4, pH 6.0, containing 8 M guanidinium·HCl. Spectra of the denatured complexes were recorded, protein was removed by ultrafiltration with a Microcon (10 min, 4 °C) and spectra of the filtrates were recorded immediately after centrifugation.

Mass Spectra of Inactivated Complexes. A portion of the washed samples, before the denaturation step described in the previous section, was washed an additional two times in 1.25 mM NH_4HCO_3, pH 7.8, and concentrated to ∼25 μL. A 10-μL sample was diluted to 50:50:0.05 (H_2O:acetonitrile:formic acid) and analyzed on a Micromass Quattro II triple quadrupole mass spectrometer by negative ion electrospray ionization mass spectrometry (ESI−MS) for the flavin adducts and by positive ion ESI−MS for protein modification. Samples were infused at 0.6 mL/h at desolvation temperatures of 80 °C and 150 °C, respectively. Parameters for negative ion mode were as follows: capillary 2.94 kV, cone 20−35 V, extractor 3 V. The corresponding values in positive ion mode were 3.5 kV, 30 and 3 V. Scans were typically performed in the range of *m*/*z* 150 to 1200 at a rate of ∼200 amu/s. Parallel large scale inactivations in H_2O and D_2O were performed as described previously.[21]

Product Analysis by GC and GC−MS. Flavin-bound IDI-2 (0.4−50 μM) was added to 40 mM HEPES buffer, pH 7.0, containing 2 mM $MgCl_2$, 2 mM NADPH, and 200−500 μM analogue to a final volume of 400 μL. The mixture was incubated (2 min, 2 h) at 37 °C. A 75-μL ice-cold solution of 533 mM glycine, pH 10.5, containing 5.33 mM $ZnCl_2$ was added (final pH 9.7), and the mixture was quickly placed on ice. IDI-2 was removed by ultrafiltration (Microcon YM-30, Millipore; 13 400 × *g*; 4 °C). The filtrate was transferred to glass test tube together with 5 μL (80 units) of calf alkaline phosphatase

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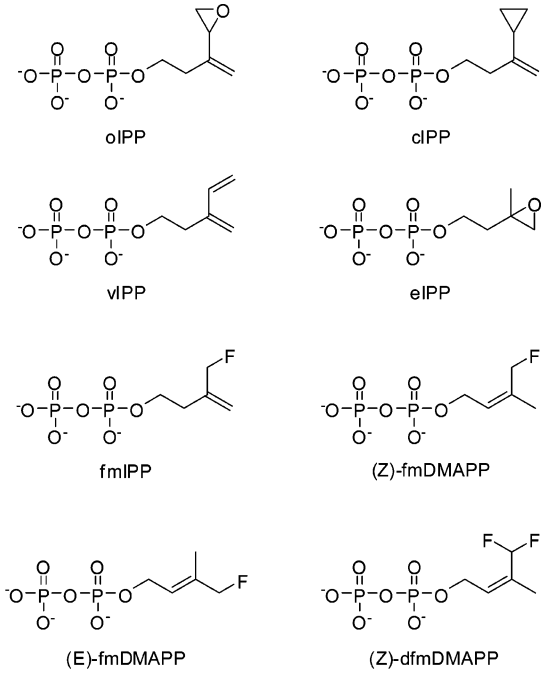

Figure 1. Structures of IPP and DMAPP analogues (racemates of oIPP and eIPP were used as inhibitors).

(Sigma) and incubated for 1 h at 37 °C. Approximately 0.4 g of NaCl was added, and the aqueous phase was extracted three times with 250 μL *tert*-butyl methyl ether. The combined extracts were concentrated to less than 25 μL with a stream of dry nitrogen. A 1-μL portion of the extract was analyzed by gas chromatography on a 30 m × 0.252 mm DB-5 capillary column (J&W Scientific). Gas chromatography was performed on a HP 6890 GC with FID detection or a HP 5971A GC-MSD with either electron impact or chemical ionization. A column temperature of 45 °C was employed for the fluorinated and diene analogues. An initial temperature of 50 °C (5 min) was followed by a temperature gradient to 150 °C at 2 °C/min was used for the cyclopropylcarbinyl and epoxycarbinyl analogues. The mass spectra of eluted peaks were compared with authentic samples. Specific assay conditions for reactions employed in rate calculations were as follows: 200 μM cIPP, 0.4 μM enzyme-FMN, 5 min assay; 200 μM vIPP, 2.5 μM enzyme-FMN, 2 min assay; 400 μM fmIPP, 20 μM enzyme-FMN, 10 min assay; 500 μM (*Z*)-fmDMAPP, 10 μM enzyme-FMN, 15 min assay; 400 μM (*Z*)-dfmDMAPP, 50 μM enzyme-FMN, 2 h assay.

Results

Kinetic Studies. IPP and DMAPP analogues (Figure 1) were tested as reversible and irreversible inhibitors of *T. thermophilus* IDI-2 under aerobic conditions at 37 °C. Irreversible loss of activity was measured by preincubation of NADPH-reduced flavoenzyme with inhibitor, followed by dilution into assay buffer containing saturating [1-^{14}C]IPP to determine residual activity. Enzyme incubated with oIPP, vIPP, or fmIPP showed concentration and time-dependent inhibition (Figures S1 and S2 of the Supporting Information). Extended incubation of enzyme in the presence of these analogues reduced isomerase activity by >95%. In comparison, IDI-2 incubated in the absence of inhibitor was fully active over a 2 h period. NADPH was essential for time-dependent inactivation, consistent with a catalytic requirement for reduced flavin.[12] The presence of IPP in the preincubation mixture was found to protect the enzyme from irreversible inactivation (Figure S3 of the Supporting Information). The concentration-dependent first-order rate constants for inactivation were fit to a saturation model to determine the kinetic parameters for inactivation (Table 1). vIPP and oIPP rapidly inactivated IDI-2, while inactivation by fmIPP was slower.

Table 1. Kinetic Parameters for Irreversible and Reversible Inhibition of *T. thermophilus* IPP Isomerase

inhibitor	k_I (min^{-1})	K_I (μM)	k_I/K_I (s^{-1} mM^{-1})
	irreversible		
oIPP[a]	0.37 ± 0.07	1.4 ± 0.3	4.4
vIPP	1.2 ± 0.1	8 ± 2	2.5
fmIPP	0.044 ± 0.002	7.4 ± 0.9	0.1
eIPP	0.041 ± 0.01	48.6 ± 8.2	0.014
	reversible		
cIPP		54 ± 6	
(*Z*)-fmDMAPP		12 ± 3	
(*Z*)-dfmDMAPP		383 ± 35	

[a] From ref 21.

Previous work had shown that millimolar concentrations of eIPP inactivated IDI-2 from *Methanocaldococcus jannaschii*.[19] We found that the epoxide also inhibited IDI-2 from *T. thermophilus* in a concentration and time-dependent manner, but at micromolar concentrations (Figure S4 of the Supporting Information). In contrast to the other analogues, eIPP appeared to reversibly inhibit IDI-2 in the presence of oxygen but was an irreversible inhibitor under anaerobic conditions. (*Z*)-fmDMAPP, (*Z*)-dfmDMAPP and cIPP were competitive reversible inhibitors (Table 1). IDI-2 slowly lost activity upon extended incubation with (*Z*)-fmDMAPP, which probably resulted from enzyme catalyzed isomerization to fmIPP, followed by irreversible inhibition by the IPP analogue.

UV−Vis and Mass Spectrometric Analysis of Inactivated Complexes. The time-dependent inhibition profiles exhibited by oIPP, vIPP, fmIPP, and eIPP analogues are consistent with inactivation of IDI-2 by covalent modification, as was previously demonstrated for IDI-1 by vIPP, fmIPP, and eIPP.[14,15,25] Positive ion electrospray mass spectrometry was used to detect potential modifications of amino acid residues in the protein. All of the deconvoluted positive ion ESI spectra gave masses within experimental error of 38 098 Da, the mass measured for apo IDI-2. Thus, the IDI-2 protein was not covalently modified by oIPP, vIPP, fmIPP, or eIPP.

UV−visible spectra for IDI-2 treated with NADPH, followed by IPP or an irreversible inhibitor and then washed to remove excess inhibitor under aerobic conditions, are shown in Figure 2A. The spectrum of the control sample incubated with IPP is identical to that reported for the oxidized enzyme complex.[12] The spectra of the flavoenzyme incubated with the inhibitors indicated that the protein was protected from reoxidation. The enzyme complexes were then denatured with guanidinium·HCl to release the flavin, followed by ultrafiltration to remove the protein. Spectra of the modified flavins obtained from the inactivated complexes are shown in Figure 2B. Those from enzyme treated with vIPP or oIPP have a peak near 370 nm and a shoulder near 310 nm, which is similar to spectra of the "pseudobase" state of 5-alkyl flavoquinonium species.[26,27] In

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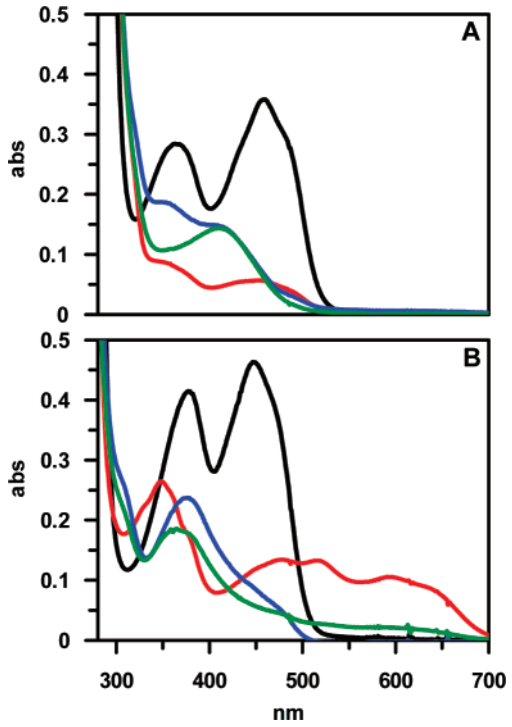

Figure 2. UV−visible absorption spectra for IDI-2 incubated with IPP (black line), oIPP (blue line), fmIPP (red line), and vIPP (green line). Spectra were measured after incubation and ultrafiltration (Part A) and following subsequent denaturation and ultrafiltration (Part B).

contrast, the modified flavin from treatment with fmIPP had peaks at 350, 480, 520, and 600 nm consistent with a stable neutral semiquinone.[28,29] A small signal for a neutral semiquinone is also observed in various spectra for the vIPP and oIPP adducts. Both the neutral semiquinone and "pseudobase" species were reported as intermediates in the oxidative degradation of N5 alkyl-dihydroflavins.[27−29] Interestingly, the semiquinone-like species derived from protein inactivated eIPP was seen after both aerobic washes and denaturation (Figure S6 of the Supporting Information). The covalent flavin·eIPP adduct was unstable and decomposed to oxidized flavin during prolonged incubation on ice (data not shown). The inactivated enzymes were also denaturated at low pH (<1). Protein inactivated with fmIPP or vIPP yielded similar spectra with a shoulder at 305 nm (Figure S7 of the Supporting Information). A 305 nm signal was observed in the spectrum of N5 ethyl-dihydroflavin in 6 N HCl.[26] In contrast, the spectra of C4a alkyl dihydroflavins have a peak at 380−390 nm at low pH. The spectra from control samples of IDI-2 and IPP were identical to the spectrum of free oxidized FMN.

Spectra were also obtained for enzyme complexes with reversible inhibitors. Interestingly, the UV−visible spectrum for enzyme·$FMNH_2$·(*Z*)-fmDMAPP closely resembles that of enzyme·$FMNH_2$·IPP, while the spectrum of reduced IDI-2 inhibited with cIPP is identical to enzyme·$FMNH^-$ (Figure S8 of the Supporting Information). The fluorinated analogue also stabilizes the neutral semiquinone flavin state during the oxidation and reduction processes, as has been previously found with the native substrate.[12]

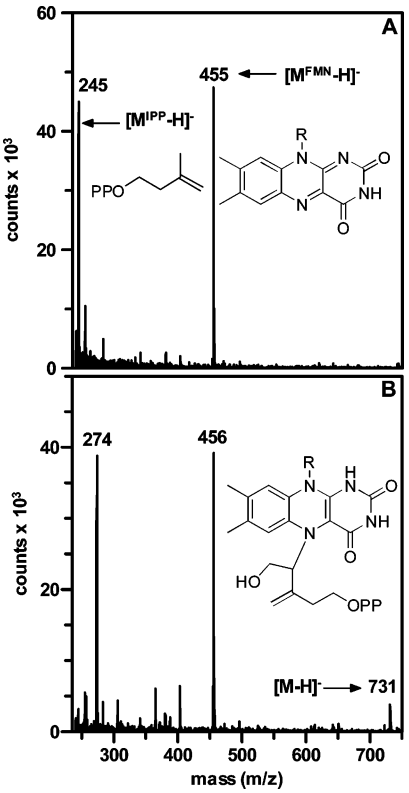

Figure 3. Negative ion ESI−mass spectra. Part A, IDI-2 incubated with IPP. Peaks at *m*/*z* 455 and *m*/*z* 245 correspond to unmodified FMN and IPP, respectively. Part B, IDI-2 inactivated with oIPP. The peak at *m*/*z* 731 corresponds to an oIPP-flavin adduct.

Negative ion electrospray mass spectrometry was used to establish the existence of flavin-inhibitor adducts. We previously reported that formation of a covalent oIPP-flavin adduct upon irreversible inhibition of IDI-2 was detected by reversed phase-HPLC and LC-mass spectrometry.[21,22] Attempts to detect similar flavin-inhibitor adducts by reversed phase-HPLC from protein inhibited with vIPP and fmIPP only gave peaks with the same retention time as free FMN, but which were 10-fold less intense than those of the IPP control. Since the conditions required to release the tightly bound flavin from the *T. thermophilus* protein are somewhat harsh and could result in decomposition of the adduct, we directly infused inactivated enzyme into the inlet of the mass spectrometer. Control experiments were conducted with enzyme treated with IPP and oIPP. Negative ion ESI−MS of enzyme incubated IPP and oIPP and washed via ultrafiltration under aerobic conditions are shown in Figure 3. Enzyme treated with IPP (part A), washed at pH 7.8, and resuspended in solution at pH 3 gave a peak for oxidized FMN at *m*/*z* 455 and for IPP at *m*/*z* 245. Similar treatment of protein inhibited with oIPP gave a peak at *m*/*z* 731 for a flavin-oIPP adduct and a fragment ion for the flavin unit at *m*/*z* 456 (part B).

Direct infusion studies were performed with enzyme inactivated by fmIPP, vIPP, eIPP, cIPP, and (*Z*)-dfmIPP. The mass spectrum of enzyme incubated with fmIPP in the absence of NADPH gave peaks for oxidized flavin at *m*/*z* 455 and the analogue at *m*/*z* 263 (Figure 4B). When NADPH was added to the incubation mixture, peaks were seen for the flavin-inhibitor adduct at *m*/*z* 701 along with fragment ions for the inhibitor unit at *m*/*z* 244 and the reduced flavin unit at *m*/*z* 456 (Figure 4A). The signal at *m*/*z* 701 shows that the flavin-analogue adduct forms with release of fluoride. Homolytic fragmentation of the

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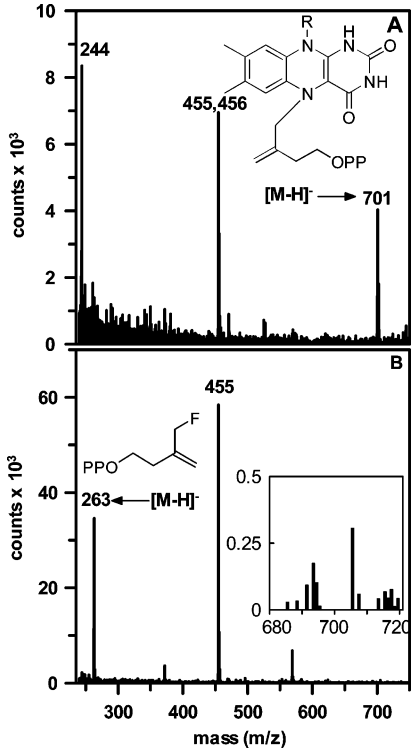

Figure 4. Negative ion ESI−mass spectra for IDI-2 incubated with fmIPP the presence (Part A) and absence (Part B) of NADPH. The low cone voltages used for ionization populate (Part A) an adduct (*m*/*z* 701), its fragmentation peaks (*m*/*z* 244 and *m*/*z* 456), and some residual oxidized flavin (*m*/*z* 455). Under high cone voltages, the *m*/*z* 244 and *m*/*z* 456 peaks predominate in mass spectra for incubations with fmIPP in the presence of NAPDH (data not shown).

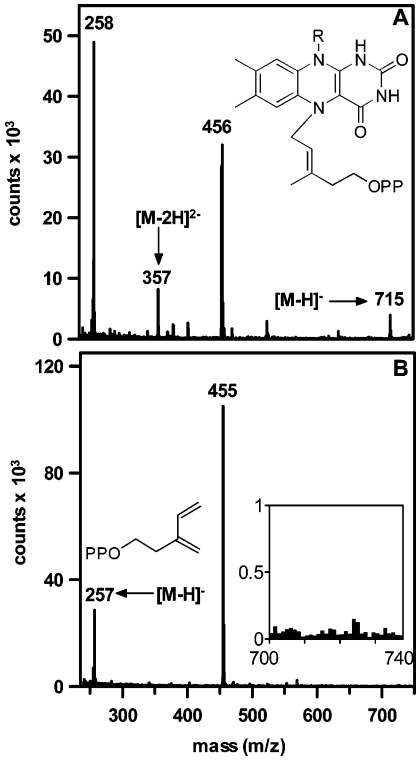

Figure 5. Negative ion ESI−mass spectra for IDI-2 incubated with vIPP in the presence (Part A) and absence (Part B) of NADPH.

adduct accounts for the two additional peaks. When fmIPP was replaced with [4-^{13}C]fmIPP in the inactivation experiment, the peak for the adduct increased by one mass unit from *m*/*z* 701 to *m*/*z* 702, and the peak for the isoprenoid fragment, from *m*/*z* 244 to *m*/*z* 245. The peak for the flavin fragment remained at *m*/*z* 456. In a related set of experiments, the mass spectrum of enzyme incubated with vIPP in the absence of reducing agent gave peaks at *m*/*z* 455 and 257 from oxidized flavin and the analogue, respectively (Figure 5B). Enzyme inactivated in the presence of NADPH gave peaks at *m*/*z* 715 for the flavin-inhibitor adduct and *m*/*z* 258 and 456 for the flavin and inhibitor fragments, respectively (Figure 5A). These masses show that a single proton or hydrogen is added to the inhibitor during inactivation of the enzyme. Furthermore, we observed 1−4 mass increases in the size of the adduct when the inactivation was performed in D_2O and the subsequent washes were in H_2O. Incorporation of 1−3 solvent deuterium atoms into the isoprenoid moiety is consistent with the reversible isomerization of vIPP to vDMAPP, which is supported by GC studies described below. Addition of the fourth deuterium occurs during the step that leads to inactivation. The distribution of peaks shows that the [$^2H_4$]FMNH$_2$·vIPP species predominates over the species with 1−3 deuterium atoms (Figure S9). The NADPH requirement for inactivation by fmIPP and vIPP demonstrates that formation of the flavin·inhibitor adducts occurs with irreversible inhibition of the enzyme. Incubation with eIPP in the presence of NAPDH gave peaks at *m*/*z* 718 and 719 *m*/*z*, consistent with formation of a flavin·EIPP adduct (Figure S10 of the Supporting Information). No flavin or protein adducts were seen for incubations with NADPH and cIPP or (*Z*)-dfmDMAPP.

Isomerization of Analogues. With the exception of eIPP, the analogues in this study are also potential substrates for IDI-2. Initial attempts to detect isomerization by proton NMR, which we used successfully to measure isomerization of cIPP to cDMAPP,[21] were unsuccessful because isomerization competed with inactivation. Instead we used a more sensitive GC-based assay. After incubation of IDI-2 with the inhibitors and NADPH, the diphosphate moieties in the inhibitor and putative isomerized products were hydrolyzed to their corresponding alcohols with alkaline phosphatase, and the samples analyzed by GC or GC−MS. Gas chromatograms for fmIPP and vIPP are shown in Figure 6, parts A and B, respectively. Each panel shows chromatograms for authentic samples of dephosphorylated substrate (red) and product (green) and for samples after incubation of substrate with IDI-2 (blue). The identities of alcohols for the signals observed in the enzyme−substrate mixture were confirmed by GC−MS (Figures S11−S13 of the Supporting Information).

Isomerizations of (*Z*)-fmDMAPP to fmIPP, (*Z*)-dfmDMAPP to dfmIPP, and cIPP to cDMAPP were also detected (Figures S14−S20 of the Supporting Information). We were unable to detect a product from isomerization of oIPP in incubations with up to 150 μM IDI-2. Although we did not have an authentic sample of oDMAPP for control experiments, it is unlikely that the isomeric alcohols produced by hydrolysis of oIPP and oDMAPP have identical retention times. Rather, the absence of turnover prior to inactivation is consistent with our inability to detect incorporation of deuterium into the oIPP-flavin adduct from incubations in D_2O[21] and the fast rate of inactivation by oIPP. This behavior is in contrast to vIPP, which isomerizes at a rate faster than inactivation and incorporates multiple deuterium atoms into the vIPP-FMN adduct.

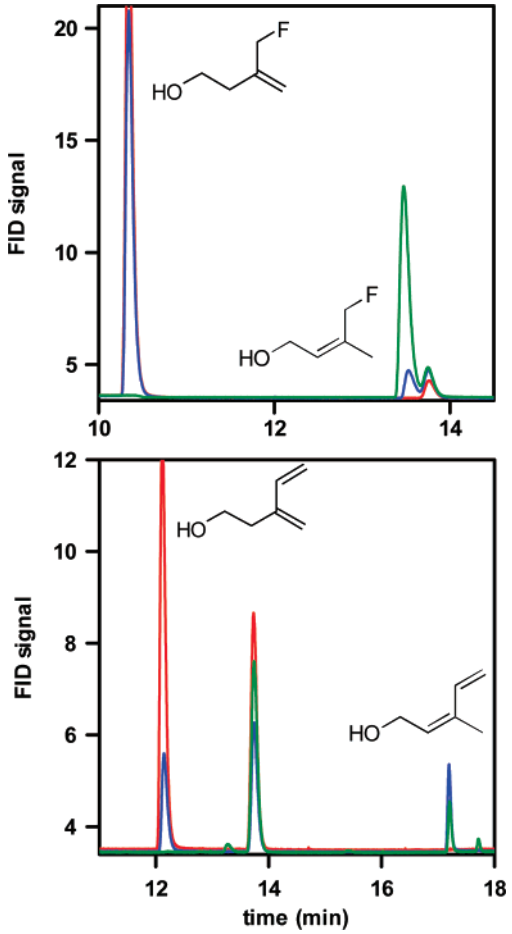

Figure 6. GC analysis of products from incubations of IDI-2 with fmIPP (Part A) and vIPP (Part B). Traces for authentic samples of substrates (red line) and products (green line) and for isolates following incubation of the substrates with IDI-2 (blue line). The identities of compounds attributed to the peaks were confirmed by electron impact mass spectrometry. The signal at 13.7 min is from an impurity in *tert*-butyl methyl ether used during extractions.

We estimated the rates of isomerization for the analogues from GC and GC−MS data (Table 2). cIPP and vIPP are good substrates for IDI-2. In contrast, the fluorinated analogues isomerized much more slowly. Comparisons of rates of isomerization for IPP with those for fmIPP, (*Z*)-fmDMAPP and (*Z*)-dfmDMAPP indicate an approximate 30- to 100-fold decrease each time a hydrogen atom was replaced by fluorine. GC−MS analysis of the products from cIPP shows that cDMAPP underwent spontaneous hydrolysis (Figures S15 and S16 of the Supporting Information) as previously observed by NMR.[21] Extended reaction times for incubations with up to 50 μM enzyme indicate that equilibrium was rapidly achieved between cIPP and cDMAPP, followed by slow hydrolysis of the allylic isomer. No isomerization was seen in the absence of a reducing agent (Figure S15).

Discussion

Several mechanisms have been suggested for the isomerization of IPP to DMAPP catalyzed by IDI-2. Recent attention has focused on the hydrogen atom addition/abstraction and the protonation-deprotonation models (Scheme 1).[10−13,18,19] The addition/abstraction mechanism involves substrate and flavin radicals as intermediates, is consistent with the strict requirement for reduced flavin, and accounts for the lack of activity with 5-deazaFMN.[11,30] Potentiometric studies indicate that IDI-2 stabilizes the neutral semiquinone flavin state[12,30] although at this time there is yet no compelling evidence for a radical species during catalysis. The protonation−deprotonation mechanism is chemically identical to the established mechanism for isomerization by IDI-1. The recent report that eIPP functions is an irreversible inhibitor of IDI-2 from *M. jannaschii*, albeit at millimolar concentrations, is consistent with a mechanism based on substrate protonation.[19] In addition, we, along with Liu and co-workers, have suggested, based on redox potentials, the possibility of a protonation−deprotonation mechanism that includes a transient single electron transfer to the carbocationic intermediate.[12,30]

Table 2. Specific Activities for Isomerization of Substrate Analogues[a]

inhibitor	SA (μmol/min·mg)	$SA^{analogue}/SA^{IPP}$
cIPP	0.19 ± 0.01	0.7
vIPP	0.047 ± 0.003	0.17
fmIPP	0.0044 ± 0.0004	0.02
(Z)-fmDMAPP	0.0036 ± 0.003	0.01
(Z)-dfmDMAPP	0.000096 ± 0.000019	0.0003

[a] Assay conditions are described in Experimental Procedures.

Studies with oIPP and cIPP failed to detect radical intermediates by the cyclopropylcarbinyl-homoallyl "radical clock" rearrangement. Instead, cIPP is converted to cDMAPP without competing homoallylic rearrangement or inactivation of the enzyme. NMR measurements of the isomerization,[21] along with more sensitive GC assays at high enzyme concentrations, show that equilibration between cIPP and cDMAPP is established rapidly, followed by nonenzymatic hydrolysis of the allylic diphosphate. A first-order rate constant of $\sim 2 \times 10^7$ sec^{-1} at 25 °C anticipated for rearrangement of a tertiary cyclocarbinyl radical to the corresponding homoallylic species places a lower limit on the lifetime of a putative radical intermediate of ~35 ns.[31] Although the rate of rearrangement for the putative enzyme-bound radical may be slower than the competing hydrogen atom abstraction to form cDMAPP, substrate and flavin radical intermediates would be formed repeatedly as cIPP and cDMAPP are turned over. The failure to observe any species or adduct derived from a radical rearrangement upon extended incubation indicates that cyclopropylcarbinyl radicals are not formed or the rate for abstraction is 10^2-10^3 times faster than rearrangement. In contrast, the cyclopropyl group in cIPP would facilitate isomerization to cDMAPP by stabilizing the tertiary carbocation produced upon protonation of the double bond in a protonation-deprotonation mechanism.[32]

In contrast to cIPP, oIPP rapidly inactivates IDI-2 with concomitant formation of a flavin adduct. Irreversible inhibition of the enzyme involves activation of the inhibitor by protonation at oxygen in the epoxide moiety, followed by alkylation of the flavin cofactor at N5.[21] Furthermore, there is no evidence for isomerization of oIPP prior to alkylation of the flavin. The contrasting abilities of the cyclopropyl and oxiranyl groups to stabilize adjacent carbocations[33] accounts for the observed rapid

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turnover of cIPP through a protonation-deprotonation mechanism and the observed lack of any oIPP turnover prior to inactivation. Since the rate constant for the radical clock rearrangement of tertiary epoxycarbinyl radicals is $\sim 3 \times 10^{10}$ s^{-1},[34] if an epoxycarbinyl radical were formed, we can estimate that its lifetime is <20 fs. In addition, we found that eIPP is a considerably more potent irreversible inhibitor of *T. thermophilus* IDI-2 than was originally reported for the *M. jannaschii* enzyme.[19] The mechanism for inactivation by eIPP is expected to be similar to that seen for oIPP,[21] with reduced flavin acting as a nucleophile during adduct formation. The absence of protein adducts in the presence of oxidized flavin suggests that either the bound analogues are effectively protected from potential nucleophiles by the flavin and/or that the reduced cofactor state is required for oxiranyl group activation as well.

fmIPP is a substrate for and an irreversible inhibitor of IDI-2. The enzyme is inhibited by alkylation of the flavin cofactor by fmIPP with the concomitant loss of fluoride. fmIPP also irreversibly inhibits IDI-1, in this case by alkylation of the sulfhydryl group in an active site cysteine residue.[25] Both reactions most likely involve nucleophilic displacement of the allylic fluorine, by $FMNH^-$ for IDI-2 and by the cysteine thiolate in IDI-1. (*Z*)-fmDMAPP and (*Z*)-dfmDMAPP do not appear to be irreversible inhibitors IDI-2, but are slowly isomerized to the corresponding IPP derivatives. Inhibition by fmIPP and not by (*Z*)-fmDMAPP is somewhat puzzling and may reflect the conformational restrictions in the allylic analogue not present in fmIPP. Preliminary studies indicate that (*E*)-fmDMAPP, like fmIPP, is both a substrate and an irreversible inhibitor. Incubation of (*E*)-fmDMAPP with the enzyme resulted in a new peak in the gas chromatogram that contains a mass fragmentation pattern similar to fmIPP, but elutes with a different retention time. Time-dependent enzyme inhibition and a UV−visible absorption spectrum similar to that observed for eIPP studies suggest the formation of a labile flavin-inhibitor adduct.

The effects of replacing hydrogen in the methyl groups of IPP and DMAPP by fluorine on the rate of isomerization do not provide definitive evidence for either mechanism. Unfortunately, there are no good models to permit a quantitative evaluation of the effect of β-fluorine substituents on the stability of a tertiary radical or carbocation. One would anticipate a tertiary carbocation is destabilized when β-hydrogen atoms are replaced by fluorine because of the powerful electron-withdrawing effect of fluorine. For example, mono- and difluoromethyl derivatives of dimethylallyl methanesulfonate, which solvolyze through allylic carbocationic intermediates, are 770- and 17 000-fold less reactive, respectively, than the parent compound.[20] Fluorine has a similar destabilizing effect on radicals. For example, the bond dissociation energy for the C−H bond in 1,1,1-trifluoroethane is 6.2 ± 1.4 kcal/mol higher than for ethane, which corresponds to a ~35 000-fold difference in rates for removal of the hydrogen atom at 25 °C.[35] The 30- to 100-fold reduction in the rate we saw for isomerization by IDI-2 for each substitution of hydrogen by fluorine could be accommodated by either mechanism.

There are several reports of irreversible inactivation of flavoenzymes by alkylation of the cofactor[36−42] and flavin adducts have been implicated as intermediates during catalysis.[43−46] Modifications have been reported at N5, C4a, and C6 in the isoalloxazine nucleus. These adducts have distinctive spectra and different susceptibilities to degradation depending on the site of modification.[26,28,29] On the basis of these observations, the flavin adducts we found following irreversible inhibition of IDI-2 are modified at N5. The inability of 5-deazaFMN to serve as an alternate cofactor for the enzyme suggests that N5 plays an important role during isomerization. In a protonation−deprotonation mechanism, the flavin could stabilize the tertiary carbocationic intermediate electrostatically and/or function as a general acid for the protonation step. Both our group[12] and Liu and co-workers[30,47] have suggested that reduced flavin may act as the proton donor for IPP isomerization. Our results with epoxy, epoxycarbinyl, and cyclopropylcarbinyl analogues indicate that an active site proton is required for isomerization and inhibition. A proton at N5 of the flavin is an attractive possibility. The 5,5-zwitterion of reduced flavin has been implicated in acid/base chemistry during catalysis,[48] but we do not have any evidence that this tautomer is involved in catalysis. It has also been suggested that the proton at N5 in the 1,5-tautomer could function as a general acid,[30,47] though it is unclear if the enzyme could lower its pKa sufficiently to allow it to protonate the carbon−carbon double bond in the substrate.[48]

Conclusions

We have found that a variety of fluorocyclopropyl and diene IPP analogues are alternate substrates for IDI-2. A subset of these compounds and epoxide analogues are also irreversible inhibitors. The behavior of these compounds is very similar to what we previously found for IDI-1. These comparisons lead us to propose a mechanism for the reaction that incorporates substrate protonation−deprotonation. Although there is no experimental evidence to support a transient single electron transfer from reduced flavin to the carbocationic intermediate, redox potentials for the *tert*-butyl radical and flavin semiquinone suggest the possibility.[12,30,49] At this point, the data are not sufficient to resolve the mechanistic details of the reaction.

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Supporting Information Available: Kinetic plots for inactivation of IDI-2 by irreversible inhibitors and IPP-mediated protection of inactivation. UV−visible spectra of E-FMN_{ox}-analog complexes and spectra of inactivated complexes in 5% trifluoroacetic acid. UV−visible spectra and negative ion ESI−MS spectra for eIPP-inactivated enzyme. ESI−MS for vIPP incubated with enzyme in deuterated solvent. Spectra of reduced enzyme complexed with reversible inhibitors. GC−MS spectra for isomerizations of fmIPP and vIPP. GC and GC-MS chromatograms for isomerizations of fmDMAPP to fmIPP, dfmDMAPP to dfmIPP, cIPP to cDMAPP, NADPH dependence of cIPP isomerization, and cDMAPP breakdown. This material is available free of charge via the Internet at http://www.pubs.acs.org.

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