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Escherichia coli Type I Isopentenyl Diphosphate Isomerase: Structural and Catalytic Roles for Divalent Metals

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Abstract: Isopentenyl diphosphate isomerase (IDI) catalyzes the essential conversion of isopentenyl diphosphate (IPP) to dimethylallyl diphosphate (DMAPP) in the mevalonate entry into the isoprenoid biosynthetic pathway. Two convergently evolved forms of IDI are known. Type I IDI, which is found in Eukarya and many Bacteria, catalyzes the isomerization of IPP and DMAPP by a protonation−deprotonation mechanism. The enzyme requires two divalent metal ions for activity. An X-ray structure of type I IDI from crystals soaked with (*N*,*N*-dimethylamino)-1-ethyl diphosphate (NIPP), a potent transition-state analogue for the carbocationic intermediate in the isomerization reaction, shows one of the metals in a His_3Glu_2 hexacoordinate binding site, while the other forms a bridge between the diphosphate moiety of the substrate and the enzyme (Wouters, J.; et al. *J. Biol. Chem.* **2003**, *278*, 11903). Reconstitution of metal-free recombinant *Escherichia coli* type I IDI with several divalent metals—Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, and Cd^{2+}—generated active enzyme. Freshly purified IDI contained substoichiometric levels of a single metal ion, presumably bound in the hexacoordinate site. When NIPP was added to the disruption and purification buffers of enzyme, the purified protein contained 0.72 equiv of Mg^{2+}, 0.92 equiv of Zn^{2+}, and 0.10 equiv of Mn^{2+}. These results are consistent with a structure in which Mg^{2+} facilitates diphosphate binding and Zn^{2+} or Mn^{2+} occupies the hexacoordinate site.

Isoprenoid compounds comprise the most diverse family of molecules found in nature. A few representative classes include sterols (membrane structure, hormones), carotenoids (photoprotection, vision), dolichols (cell wall and glycoprotein biosynthesis), ubiquinones (respiration), and prenylated proteins (signal transduction, nuclear membrane structure).[1] The carbon skeletons of isoprenoid molecules are constructed from two simple five-carbon building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). There are two pathways for biosynthesis of IPP and DMAPP.[2] In Eukarya and a few Eubacteria, IPP is synthesized from three molecules of acetyl coenzyme A by the well-known mevalonate (MVA) pathway.[3] The conversion of IPP to DMAPP, catalyzed by isopentenyl diphosphate isomerase (IDI), is a required step for isoprenoid compounds synthesized by the MVA pathway. Isoprenoid biosynthesis in Archaea follows a closely related pathway, although not all of the intermediates between MVA and IPP have been firmly established. In plant chloroplasts, most eubacteria, cyanobacteria, and apicoplast-type protozoa, IPP and DMAPP are synthesized from glyceraldehyde-3-phosphate and pyruvate by the methylerythritol phosphate (MEP) pathway.[4] In the final step, IPP and DMAPP are both produced during the reduction of hydroxydimethylallyl diphosphate. Although IDI activity is not required for isoprenoid biosynthesis via the MEP pathway, most bacteria have a functional IDI, presumably to balance the pools of IPP and DMAPP.[5,6]

Two convergently evolved forms of IDI are known. Type I IDI was discovered in the 1950s and is found in Eukaryotes and many Bacteria. The gene for type I IDI has been cloned from a variety of sources, including humans,[7] *Saccharomyces cerevisiae*,[8] *Escherichia coli*,[6] and *Rhodobacter capsulatus*.[9] The enzyme requires a divalent metal for activity, and mechanistic studies support an antarafacial protonation−deprotonation mechanism for the isomerization reaction (see Scheme 1).[10] Among the experiments which support a protonation−deprotonation mechanism for type I IDI are studies with transition-state analogues[11] and active-site-directed irreversible inhibitors.[12] Covalent modification of amino acid residues by the inhibitors, supported by site-directed mutagenesis, suggests that C67 (*E.*

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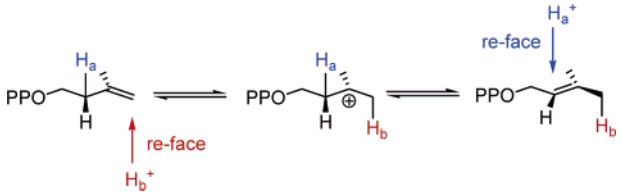
Scheme 1. Mechanism for Isomerization of IPP to DMAPP by Type I IDI



coli numbering) and E116 are key parts of the protonation−deprotonation machinery. Type II IDI, first reported in 2001,[13] is found in Archaea,[14,15] cyanobacteria,[16] and some Gram-positive bacteria.[17] This enzyme requires flavin mononucleotide, reduced nicotinamide adenine dinucleotide phosphate, and divalent metal ions for activity.[13] The mechanism for type II IDI has not been established.

X-ray structures of the *E. coli* type I IDI·inhibitor complexes revealed the presence of two metals, which were assigned as Mn^{2+} and Mg^{2+} on the basis of differences in the intensities of electron density maps.[18,19] The more dense metal, presumably Mn^{2+}, is coordinated to H25, H32, H69, both side-chain carboxylate oxygens of E114, and one of the side-chain oxygens of E116 in a distorted octahedral binding site. The less dense metal, presumably Mg^{2+}, is coordinated to nonbridging oxygens at P(1) and P(2) of the diphosphate moiety of the inhibitors, an oxygen in the side-chain carboxylate of E87, the main-chain amide carboxyl oxygen of C67, and two molecules of water. We now report studies with *E. coli* IDI which show that the enzyme is activated by a wide variety of divalent metal ions and establish the preferences and locations of the divalent metals in native type I IDI.

Materials and Methods

***E. coli* Type I IDI.** *E. coli* type I IDI was obtained by modification of the procedure described by Carrigan and Poulter.[20] Four grams of cell paste from *E. coli* strain JM101/pAPHIII22,[6] grown in Luria broth (LB)/ampicillin or M9/casamino acids−glucose media (CAGM)/ampicillin minimal medium supplemented with metal ions,[20] was suspended in 20 mL of 5 mM potassium phosphate, pH 7.5, containing 10 mM β-mercaptoethanol (BME) and 1 mM phenylmethylsulfonyl fluoride and disrupted by sonication. After centrifugation at 10000*g* for 30 min, the supernatant was loaded onto a DE52-cellulose column, pre-equilibrated with 5 mM KH_2PO_4, pH 7.5, containing 10 mM BME (buffer A). The column was eluted at 2 mL/min with a linear gradient of buffer A to 100% 500 mM KH_2PO_4, pH 7.5, containing the same concentrations of BME. Fractions containing active IDI were collected and concentrated. The concentrated solution was loaded onto a Sephacryl S200 column (Amersham Bioscience), pre-equilibrated with 5 mM KH_2PO_4, pH 7.5, containing 10 mM BME. The column was eluted with a linear gradient with the same buffer. Active fractions were combined and dialyzed against 50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), pH 7.5, containing 300 mM NaCl, 0.5 mM dithiothreitol (DTT), and 20% (v/v) glycerol at 4 °C for 12 h to give 10 mg (7.6 mg/mL) of protein, which was stored at −80 °C.

IDI Assay. IDI activity was measured by the acid lability procedure.[11] Metal-free 50 mM HEPES, pH 7.0, containing 200 mM ultrapure KCl, prepared from deionized water, was treated with Chelex-100 (Bio-Rad) before addition of the following compounds to the indicated final concentrations: l mg/mL BSA, 0.5 mM DTT, 350 μM [1-^{14}C]IPP (2 μCi/μmol), and 10 mM of the indicated metal salts. The reaction was initiated by addition of enzyme, followed by incubation for 10 min at 37 °C. The reaction was quenched by addition of 0.2 mL of HCl/methanol (1:4, v/v), followed by an additional incubation for 10 min at 37 °C. Acid-labile material was extracted by vortexing with 1 mL of ligroine (bp 90−120 °C). A 0.5 mL sample of the ligroine layer was carefully removed from the upper layer and added to 10 mL of scintillation cocktail for liquid scintillation counting.

ApoIDI. All solutions were prepared with 18 MΩ deionized water and treated with Chelex. Pipet tips and Eppendorf tubes were rinsed with water treated with 50 g of Chelex/L prior to handling the apoprotein. *E. coli* type I IDI (1−5 mg) was incubated with 6 M guanidinium hydrochloride (GdnHCl) and Chelex (∼500 mg) with gentle shaking for 15−17 h at room temperature. The resulting solution was filtered through a 0.2 μm low-binding protein membrane prewashed with metal-free water to remove the Chelex resin. The filtrate was immediately loaded onto a PD-10 gel filtration column (Amersham Bioscience), pre-equilibrated with 10 mM HCl solution to remove GdnHCl. Metal-free denatured IDI was eluted with the same solution according to the manufacturer's protocol.

Reconstitution of ApoIDI. A 50 μL sample of the denatured apoIDI (∼100 μg of protein/mL) in 10 mM HCl was added to 50 μL solutions of different concentrations of the respective metal salts ($MgCl_2$, $ZnCl_2$, $MnCl_2$, $CoCl_2$, $CdCl_2$, and $NiCl_2$, atomic absorption grade, Alfa Aesar), followed by addition of 150 μL of 100 mM Tris, pH 7.4. The mixture was allowed to stand for 1 h at room temperature before assays were performed. Samples submitted for metal analysis were prepared by a similar protocol with dilution into 100 mM Tris buffer, pH 7.4, containing 50 mM NaCl. After 1 h at room temperature, the solution was centrifuged at 10000*g* for 10 min. The supernatant was loaded onto a Sephadex G25 column (Amersham Bioscience) eluted with 100 mM Tris, pH 7.4, containing 25 mM NaCl. The solution was assayed for IDI activity and analyzed for divalent metals.

Purification of IDI in Buffer Containing NIPP. *E. coli* strain JM101/pAPHIII22 cells (∼40 mg), grown in LB/ampicillin, were washed with 5 mM KH_2PO_4, pH 7.5, containing 10 mM BME, suspended in 1 mL of the same buffer containing 1 mM 2-(*N*,*N*-dimethylamino)-1-ethyl diphosphate (NIPP), and disrupted by sonication. The suspension was spun at 14000*g* for 15 min at 4 °C, and the supernatant was gently shaken for 30 min at 4 °C. The solution was loaded onto a DE52-cellulose column (Whatman), pre-equilibrated with 5 mM KH_2PO_4, pH 7.5, containing 10 mM BME. The column was eluted at 1 mL/min with a linear gradient of starting buffer to 100% 500 mM KH_2PO_4, pH 7.5, containing 10 mM BME. The eluent was concentrated with an Amicon filtration unit (10 kDa cutoff, Millipore). The filtrate was loaded onto a Sephadex G25 column, pre-equilibrated with 100 mM Tris, pH 7.4, containing 25 mM NaCl, and eluted with the same buffer. A small portion of the sample was assayed for IDI activity, and the remainder was analyzed for divalent metals.

Metal Analysis. Metal concentrations were determined by inductively coupled plasma (ICP) emission spectrometry (Perkin-Elmer 3100 XL Optima) in Prof. Dennis Winge's laboratory, Department of Biochemistry, University of Utah, or at the Chemical Analysis Laboratory (Thermo Jarrell-Ash 965 Inductively Coupled Argon Plasma), University of Georgia.

Protein Concentration. Protein concentrations were measured by UV spectroscopy, $\epsilon_{280} = 40\,340\ M^{-1}\ cm^{-1}$,[20] or using a Micro BCA Protein Assay Kit (Pierce) calibrated with a sample of IDI.

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Table 1. Mole Fraction of Divalent Metal in Reconstituted *E. coli* Type I IDI[a]

divalent metal	reconstituted IDI
Mg^{2+}	0.61 ± 0.09
Mn^{2+}	0.71 ± 0.13
Co^{2+}	0.72 ± 0.15
Ni^{2+}	0.76 ± 0.14
Cd^{2+}	0.65 ± 0.15

[a] ICP analysis for 20 metals. Concentrations of other metals were at background levels.

Results

Reconstitution of ApoIDI with Divalent Metals. Several methods were evaluated for reconstituting *E. coli* IDI with different divalent metals. Previously, apoIDI was obtained by dialysis of the protein against buffer prepared from metal-free deionized water, 50 mM Tris, pH 7.5, containing 10 mM BME and 0.5 mM *o*-phenanthroline.[20] Different concentrations of metal salts (1−100 equiv) were then added to the apoprotein. Apoprotein prepared in the same manner was also dialyzed against buffer containing Mg^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, or Cd^{2+}. However, these attempts at reconstitution gave enzyme with only 5−10% of the activity of wild-type IDI in the standard assay (data not shown). In another approach, IDI was denatured with 6 M GdnHCl.[21] The denaturated protein was treated with *o*-phenanthroline, and the buffer was replaced with fresh buffer containing 6 M GdnHCl using an Amicon filtration unit. The protein was diluted 100-fold with 50 mM Tris buffer, pH 7.0, 7.5, or 8.0, containing 1 mM DTT and 1−100 equiv of divalent metal. However, this method resulted in precipitation of IDI during the dilution step when the concentration of protein exceeded ∼200 μg/mL. Additives such as 300 mM NaCl, 10% (v/v) glycerol, 2 M urea, or 0.5 M L-arginine[22,23] were not effective in preventing precipitation. The activity of IDI reconstituted by this approach was only slightly higher than that of enzyme from the *o*-phenanthroline treatment (data not shown). The most successful approach for preparing apoIDI involved denaturation of IDI with 6 M GdnHCl/10 mM HCl, treatment with Chelex to remove metal, and removal of GdnHCl by gel filtration. The protein was refolded by dilution into pH 7.4 buffer. Some precipitation was seen when the enzyme was reconstituted with Cd^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, or Ni^{2+}, although the amount was significantly lower than observed for the other protocols. The mole fraction of each metal present in the protein is given in Table 1. Precipitation was more pronounced with Zn^{2+}. Although enough soluble protein was obtained for determining protein concentration and kinetic measurements, we were not able to get an accurate measurement of the zinc concentration.

Figure 1 compares the activities of native IDI and enzyme reconstituted with different divalent metals using the standard isomerase assay in buffer containing 10 mM $MgCl_2$. In most cases, IDI refolded in buffer containing 1 equiv of the divalent metal was more active than protein refolded in buffer containing 5 equiv of the metal. Protein refolded in metal-free buffer had ∼50% of the activity of a control sample of IDI that had not been subjected to the denaturation−refolding protocol when the samples were assayed under identical conditions in buffer containing 10 mM $MgCl_2$. No IDI activity was observed when the refolded metal-free apoenzyme was assayed in buffer free of divalent metals. When metal-free IDI was assayed in the standard buffer containing 10 mM $MgCl_2$ at short incubation times, the maximal specific activity (∼50% of wild-type enzyme) was reached within ∼10 s. Thus, IDI can rapidly incorporate Mg^{2+} from the assay buffer into both divalent metal binding sites. IDI refolded in the presence of Mg^{2+} had >80% of the activity of the control sample. The highest specific activities were seen when IDI was refolded in the presence of Co^{2+} (∼100%) and Ni^{2+} (∼200%). Interestingly, protein refolded in the presence of Zn^{2+}, thought to be the "native" metal in the hexacoordinate binding site of IDI,[20] was less active than protein refolded in the presence of the other metals. These differences were greater when IDI was refolded in 5 equiv of the metals. However, Zn^{2+} did not reduce the activity of IDI when the native protein was incubated with 1−100 equiv of $ZnCl_2$ (<100 μM) for 1 h at room temperature prior to the assay.

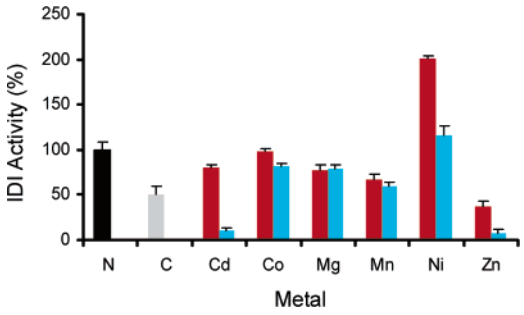

Figure 1. Catalytic activity of IDI reconstituted with different divalent metals. ApoIDI was reconstituted in the presence of 1 (red bars) and 5 (blue bars) equiv of $CdCl_2$, $CoCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, or $ZnCl_2$. Activity was measured in assay buffer containing 50 mM HEPES, pH 7.0, containing 200 mM KCl, l mg/mL BSA, 0.5 mM DTT, 350 μM [1-^{14}C] IPP (2 μCi/μmol), and 10 mM $MgCl_2$. Activities were reported relative to that of native IDI (N, black bars). The effect of Mg^{2+} in the assay buffer is measured for IDI folded in metal-free buffer (C, gray bars).

Replacement of Mg^{2+} in the Assay Buffer with Different Divalent Metals. The specific activity of IDI reconstituted with 1 equiv of different metals was also measured in assay buffer in which 10 mM Mg^{2+} was replaced by the divalent metal (Zn^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, or Ni^{2+}) used during refolding. The data are presented in Figure 2. The activity of native IDI varied substantially when the sample was pre-equilibrated and assayed in buffers containing different metals (Figure 2a), ranging from 120% for Ni^{2+} to 2% for Cd^{2+}. Similar results were seen for IDI reconstituted in metal-free buffer (Figure 2b) or buffer containing 1 equiv of the corresponding divalent metal (Figure 2c). The highest specific activities were consistently seen for Mg^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+}. Interestingly, the highest activity was observed for protein reconstituted with Ni^{2+} and assayed with 10 mM $MgCl_2$, indicating a preference for two different metals.

The specific activity of native IDI was also determined in buffers containing 50 μM to 10 mM $MgCl_2$, $ZnCl_2$, $MnCl_2$, $CoCl_2$, $CdCl_2$, or $NiCl_2$ (see Figure 3). For Mg^{2+}, Mn^{2+}, and Co^{2+}, the specific activities did not change substantially at concentrations between ∼100 μM and 1 mM. In contrast, the activity of IDI measured in the presence of Ni^{2+} increased 5-fold over the same range, while the activity in buffers containing

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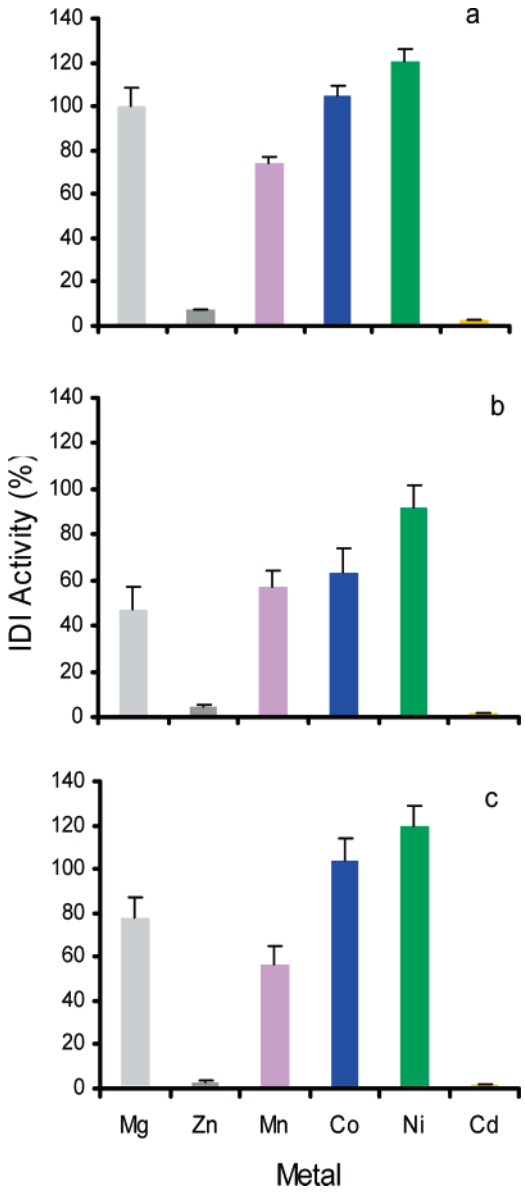

Figure 2. Activity of IDI in 50 mM HEPES, pH 7.0, containing 200 mM KCl, l mg/mL BSA, 0.5 mM DTT, 350 μM [1-^{14}C] IPP (2 μCi/μmol), and 10 mM $MgCl_2$ (light gray bars), $ZnCl_2$ (dark gray bars), $MnCl_2$ (purple bars), $CoCl_2$ (blue bars), $NiCl_2$ (green bars), or $CdCl_2$ (yellow bars), relative to native IDI in buffer containing 10 mM $MgCl_2$ (100%). (a) Native IDI. (b) IDI reconstituted in divalent metal-free buffer. (c) IDI reconstituted in 1 equiv of the corresponding divalent metal.

Zn^{2+} and Cd^{2+} dropped rapidly beginning at 100 μM Zn^{2+} and 1 mM Cd^{2+}.

Analysis of Metal Ions in the NIPP·IDI Complex. IDI was purified from *E. coli* grown in LB medium without supplementation with divalent metals. The cells were divided into two portions. One was disrupted in divalent metal-free buffer, and the other was disrupted in the same buffer containing 1 mM NIPP. The protein in both samples was purified by ion exchange and gel filtration chromatography in divalent metal-free buffers. The sample was then analyzed for metal content (Table 2). IDI from cells disrupted in buffer without NIPP contained approximately one metal atom per molecule of protein. Zinc and manganese were the most abundant, with a zinc/manganese ratio of ~10:1. Small amounts of magnesium and nickel were also detected. Although care was taken to exclude extraneous sources of metals, it is possible that the nickel in the samples came from trace amounts of Ni^{2+} commonly used in the laboratory to purify His-tagged proteins. In contrast, analysis of the protein from cells disrupted in the presence of NIPP revealed that an atom of magnesium was bound in addition to zinc (or manganese). These results are consistent with the X-ray structure of IDI·NIPP, which contained two metals.[18,19] Furthermore, the data suggest that Zn^{2+} (or Mn^{2+}) is located in the His_3Glu_2 hexacoordinate site and Mg^{2+} is coordinated to the diphosphate moiety of NIPP.

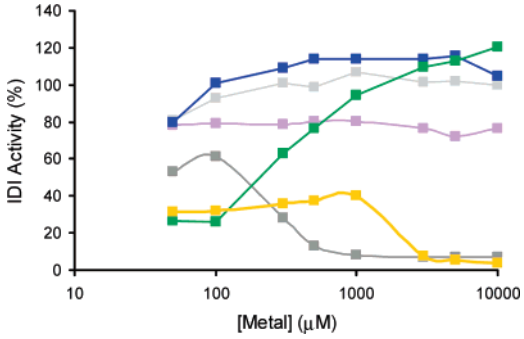

Figure 3. Semilog plot of the activity of native IDI assayed in 50 mM HEPES, pH 7.0, containing 200 mM KCl, l mg/mL BSA, 0.5 mM DTT, 350 μM [1-^{14}C] IPP (2 μCi/μmol), and different concentrations of $CdCl_2$ (yellow), $CoCl_2$ (blue), $MgCl_2$ (light gray), $MnCl_2$ (purple), $NiCl_2$ (green), or $ZnCl_2$ (dark gray), relative to native IDI in buffer containing 10 mM $MgCl_2$ (100%).

Table 2. Mole Fraction of Divalent Metals in *E. coli* Type I IDI and the NIPP·IDI Complex[a]

metal[b]	IDI	IDI·NIPP
Mg^{2+}	0.006 ± 0.002	0.72 ± 0.9
Zn^{2+}	1.02 ± 0.08	0.92 ± 0.07
Mn^{2+}	0.08 ± 0.01	0.10 ± 0.03
Ni^{2+}	0.02 ± 0.01	0.03 ± 0.02

[a] Cells were grown in LB media. [b] Concentrations for 20 metals were measured by ICP. Values corresponding to <0.01 are not shown.

Discussion

Type I IDI catalyzes the interconversion of IPP and DMAPP by an antarafacial [1.3] proton shift via protonation of the carbon−carbon double bond in either substrate, followed by deprotonation of the tertiary cationic intermediate (Figure 1). The reaction is facilitated by acidic/basic groups in the active site. Two conserved residues in IDI, C67 and E116 in the *E. coli* enzyme, were identified by covalent modification of the amino acids by active-site-directed irreversible inhibitors containing epoxide (3,4-epoxy-3-methyl-1-butyl diphosphate, EIPP),[12] allylic fluoride (3-fluoromethyl-3-buten-1-yl diphosphate, FIPP),[24] or diene (3-methylene-4-penten-1-yl diphosphate, VIPP)[25] moieties (Scheme 2). The X-ray structure of the IPP·EIPP complex shows that the oxirane ring had been opened to give a C(4) primary alcohol and a C(3) thioether with the sulfhydryl group in C67.[18] In the IPP·VIPP complex, the methylene group in the diene has been protonated, and the hydrocarbon unit is attached to the protein by a thioether linkage between C(5) of the inhibitor and C67.[25] The mechanism for covalent inactivation

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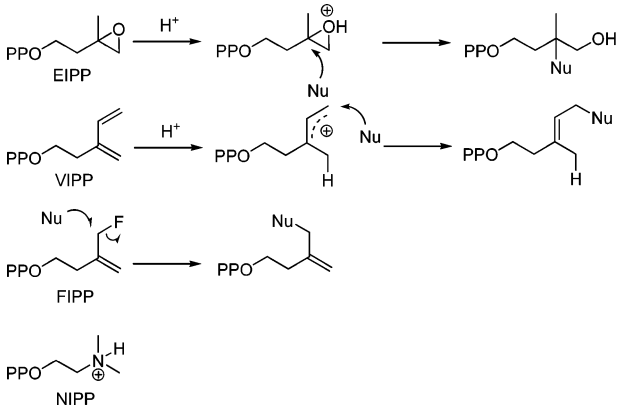
Scheme 2. Inhibitors of Type I IDI



of type I IDI by EIPP and VIPP involves protonation of the inhibitor, followed by electrophilic alkylation of the sulfhydryl group in C67. NIPP is an analogue for the putative tertiary carbocationic intermediate and is a slow, tight-binding inhibitor of IDI.[11] The structure of the IPP·NIPP complex shows that the side-chain carboxylate group of E116 is coordinated to the N−H$^+$ unit in the analogue.[18]

Several lines of evidence implicate E116 in the protonation step. Substitution of E116 by alanine abolishes activity. The proton in the N−H$^+$ unit of NIPP is hydrogen-bonded to E116.[18] The methyl group formed during protonation of VIPP is located 4 Å from the side-chain oxygen of E116.[25] Also, the C67A mutant, which does not catalyze isomerization of IPP, is irreversibly alkylated by EIPP, presumably by protonation of the oxirane ring followed by alkylation of the carboxylate moiety of E116.[19]

Type I IDI requires a divalent metal for activity. The enzyme was inactive when unfolded, stripped of metals, refolded, and assayed in divalent metal-free buffers. Activity was restored when the protein was reconstituted and assayed in buffers containing Cd^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+}. Protein refolded in divalent metal-free buffer and assayed in buffer containing $MgCl_2$ regained maximal activity within 10 s of mixing. Except for Mg^{2+}, the enzyme refolded with 1 equiv of the divalent metal was more active than enzyme refolded with 5 equiv of metal. These differences, which were particularly noticeable for Cd^{2+}, Ni^{2+}, and Zn^{2+}, may reflect interference with refolding by the metals. The activity of IDI depends on the concentration and type of divalent metal ions in the buffer. The levels of activity were substantially lower for Cd^{2+} and Zn^{2+} than for the other four metals. The activities vary ~100-fold for the six divalent metals we studied at 10 mM concentrations. This variation narrowed to only ~5-fold at 50 μM concentrations of divalent metal.

The first 30 N-terminal amino acids are disordered in the X-ray structure of metal-free *E. coli* type I IDI.[26] With a divalent metal present, this disordered region folds to give an ordered structure with the metal in a distorted octahedral binding site consisting of H25, H32, H69, one of the side-chain carboxylate oxygens of E116, and both carboxylate oxygens of E114 (Figure 4).[26] A second metal is seen in the X-ray structures of the IDI·NIPP, IDI·EIPP, and IDI·VIPP complexes in a diphosphate

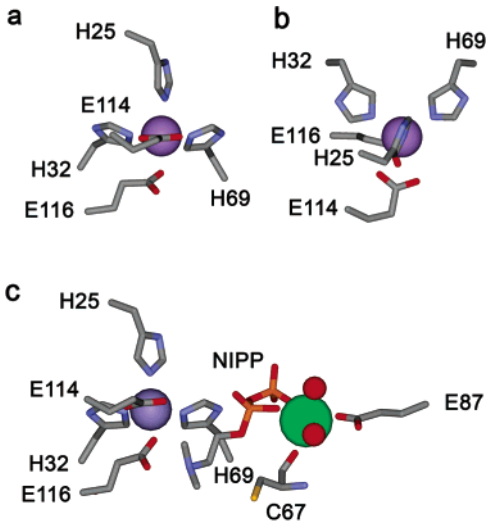


Figure 4. X-ray structure of the *E. coli* type I IDI·NIPP complex. (a) Hexacoordinate Zn^{2+} binding site viewed in the equatorial plane, showing the axial ligands H25 and E114. (b) Hexacoordinate Zn^{2+} binding site viewed along the axial ligands H25 (top) and E114 (bottom), showing the equatorial ligands H32, H69, and E114. (c) Zn^{2+} and Mg^{2+} binding sites.

binding site formed by the main-chain amide carbonyl oxygen of C67, the side-chain carboxylate of E87, the nonbridging diphosphate oxygens at P(1) and P(2) in the inhibitors, and two water molecules (Figure 4c).[18,25] Our results show that IDI is active when both sites are occupied by Cd^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+}. However, the activity is higher for combinations of Mg^{2+} and one of the other metals, suggesting that the protein prefers to bind two different metals. Differences in the electron density map for IDI·EIPP crystallized from buffer containing Mn^{2+} and Mg^{2+} indicated that Mn^{2+} was coordinated in the octahedral site and Mg^{2+} in the diphosphate site.[18]

We found that *E. coli* type I IDI can utilize a variety of divalent metals at either site. To resolve which metals were preferred by the native protein, we determined the metal content of enzyme obtained from cells grown in LB medium. *E. coli* type I IDI from cells washed and disrupted in divalent metal-free buffer and purified in divalent metal-free buffer contained 1 equiv of zinc/manganese in a ratio of ~10:1, in agreement with our previous report.[20] When the same batch of cells was disrupted in the same buffer containing NIPP and purified as described above, the IDI·NIPP complex contained an equivalent of Mg^{2+} in addition to an equivalent of 10:1 zinc/manganese. The results are in accord with the X-ray structure of the complex (Figure 4c) and demonstrate that heavier metal is in the hexacoordinate site and magnesium is in the diphosphate site.

X-ray structures of the type I IDI·inhibitor complexes help define the roles of the two divalent metals. Clearly, Mg^{2+} promotes substrate binding by forming a bridge between the diphosphate moiety and the protein. Zn^{2+} organizes the structure of the protein in the vicinity of the His_3Glu_2 site. In addition, coordination between Zn^{2+} and E116 should lower the pK_a of the side-chain carboxyl group. E116 is an essential active-site amino acid that, along with Y104, appears to be involved in protonation of the carbon−carbon double bond of IPP during catalysis. The precise mechanism of the protonation step is still uncertain. The side-chain carboxyl groups in E114 and E116 are probably in the carboxylate form in order to neutralize the net charge of the metal-coordinated His_3Glu_2 site. Thus, it

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unlikely that the side-chain carboxyl group in E116 directly transfers a proton to the double bond. Perhaps E116 facilitates proton transfer from Y104 to the substrate by stabilizing the resulting carbocation through electrostatic interactions. It is also possible that a water molecule in the active site of the enzyme·substrate complex is the source of the proton. This mechanism is suggested by the X-ray structure of the IDI·EIPP complex, where the hydroxyl group of the covalently immobilized alcohol is hydrogen-bonded to E116 and Y104.[18]

The most common Zn^{2+} binding sites in metalloproteins have tetrahedral four-coordinate or trigonal bipyramidal five-coordinate geometries, although five- or six-coordinate octahedral geometries are known.[27−29] The Zn^{2+} in type I IDI is located in a relatively uncommon six-coordinate His_3Glu_2 pocket with a distorted octahedral geometry. Thus, it is perhaps not surprising that Zn^{2+} can be replaced with metals of similar charge and size that readily form octahedral complexes. Zn^{2+} substitutions have been reported for several enzymes. In the case of astacin, the protein has a pentacoordinate trigonal bipyramidal zinc site that also binds Hg^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+} to give metalloproteins with virtually identical structures.[30] In other instances, the coordination number of the bound metal changes. For example, while zinc is tetrahedrally coordinated in human carbonic anhydrase, the Cu^{2+}, Ni^{2+}, and Mn^{2+} forms of the enzyme are five- or six-coordinate.[31] All of the metal derivatives of type I IDI we studied were active. In contrast, only the Co^{2+} and Cu^{2+} forms of astacin and the Co^{2+} derivative of carbonic anhydrase were active. These differences probably arise from different roles for the divalent metal. The mechanisms for amide bond hydrolysis or hydration of carbon dioxide require a nucleophilic water molecule in the inner coordination sphere of the metal.[30,31] In contrast, there are no changes in the ligands directly attached to Zn^{2+} in IPP isomerase during catalysis.

Given the ability of *E. coli* type I IDI to productively incorporate a variety of divalent metals, it is unclear why zinc is the metal of choice. Zinc is relatively abundant in *E. coli* grown in LB (∼100 μM), and the zinc/manganese ratio in LB (∼10:1) is similar to the ratio we found in IDI. However, zinc, and presumably manganese, is not freely available in the cytosol of *E. coli*. Outten and O'Halloran found that essentially all of the zinc in *E. coli* is sequestered and suggested that tightly regulated cytoplasmic zinc trafficking factors control the kinetics of zinc exchange between proteins.[32]

In summary, *E. coli* type I IDI requires two divalent metal atoms for activity. In its native resting form, type I IDI is a zinc metalloprotein. The divalent metal is located in a hexacoordinate His_3Glu_2 pocket, stabilizes the N-terminus of the enzyme, and is a part of the catalytic machinery for protonating the carbon−carbon double bond in IPP. In addition to zinc, the enzyme·substrate complex contains an atom of magnesium that facilitates substrate binding through the diphosphate moiety.

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