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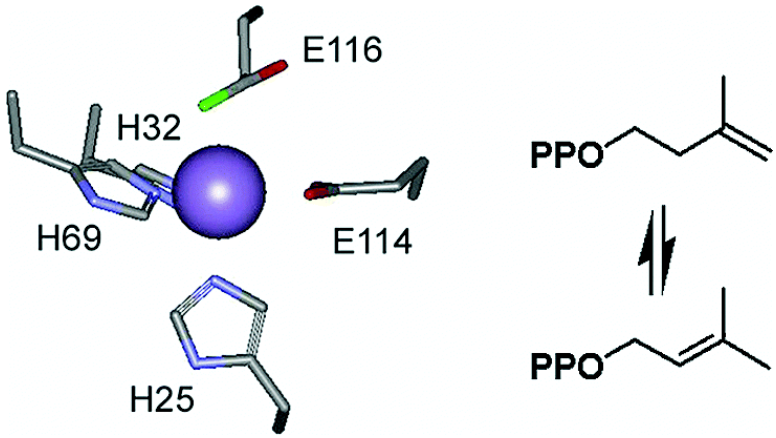

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Zinc Is an Essential Cofactor for Type I Isopentenyl Diphosphate:Dimethylallyl Diphosphate Isomerase

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Isopentenyl diphosphate:dimethylallyl diphosphate (IPP:DMAPP) type I isomerase catalyzes a required step in the mevalonate (MVA) route to isoprenoid molecules.[1] IPP isomerase is also found in some organisms that synthesize isoprenoid compounds by the methylerythritol phosphate (MEP) route,[2] although in this case IPP and DMAPP are both formed by reduction of 4-hydroxydimethylallyl diphosphate and the enzyme probably serves to balance the pools of the two C_5 intermediates for subsequent chain elongation reactions.[3] A second enzyme, type II IPP isomerase, was recently discovered.[4] This protein appears to be structurally and mechanistically unrelated to the type I enzyme.

Type I IPP isomerase catalyzes an antarafacial isomerization of IPP and DMAPP, as shown in Scheme 1.[5] The enzyme requires either Mg^{2+} or Mn^{2+} for activity.[6] The mechanism for the reaction is believed to involve protonation of the double bond, followed by elimination of a proton from the tertiary cation. Affinity labeling and site-directed mutagenesis experiments implicate two residues, C67 and E116 (*Escherichia coli* numbering), in the proton-transfer steps.[7,8] Crystal structures of *E. coli* IPP isomerase show that these two amino acids are located on either side of an active-site pocket.[9,10] In addition, E116 also forms part of an octahedral metal binding site, tentatively assigned to Mn^{2+}.[9,10] Recently, a structure was reported for IPP isomerase containing a transition-state analogue for the tertiary cation, where the C(3) carbon is replaced by a positively charged N−H ammonium unit.[11] In this structure one of the carboxylate oxygen atoms in the E116 side chain is coordinated directly to the metal ion and the other to the N−H moiety by a hydrogen bond. C67 is located on the opposite face of the analogue where it could participate in the reaction. In another structure where IPP isomerase had been inactivated with the epoxy analogue of IPP, the epoxide ring was opened to give a primary alcohol at C(4) and a thioether bond between the cysteine sulfur and C(3) of the inhibitor.[11] That structure showed two active-site metal atoms with different electron densities. The heavier metal was in the octahedral site assigned to Mn^{2+}. The lighter metal was coordinated with a nonbridging oxygen atom of P(1) and P(2) in the diphosphate moiety, a side chain oxygen in the carboxylate moiety of E87, the oxygen of the backbone carbonyl in C57, and two molecules of water. It was assigned to Mg^{2+}.

Recombinant *E. coli* Type I IPP isomerase was obtained from *E. coli* strain JM101/pAPHIII22[2] grown in a M9/CAGM minimal medium supplemented with metal ions.[12] The enzyme was purified by chromatography on DE52-cellulose and Sephacryl 200 columns.[2] Fractions containing IPP isomerase were collected, combined, and precipitated with 33% (w/v) $(NH_4)_2SO_4$. The purified protein was stored at 4 °C as an ammonium sulfate slurry in 5 mM potassium phosphate buffer, pH 7.5, containing 10 mM β-mercaptoethanol (BME). Purified protein was dissolved in the appropriate buffer to a concentration of 0.1−0.5 mg/mL prior to use. Protein concentrations were measured by UV spectroscopy using $\epsilon_{280} = 40340\ M^{-1}\ cm^{-1}$ determined by a quantitative amino acid analysis. Samples of IPP isomerase (4.0−8.0 mL, 0.1−0.5 mg/mL) in 50 mM Tris buffer, pH 7.5, containing 10 mM BME (TB) prepared from metal-free deionized water were dialyzed for 6 h at 4 °C against three changes of 1 L of TB. The metal content of the samples was determined by atomic absorption (Ca, Cd, Co, Cu, Fe, Mg, Mn, Ni, Zn) and inductively coupled plasma (Al, B, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Si, Sr, Zn) analysis. All of the metals except for Zn were at background levels relative to a control sample containing no enzyme. A portion of each sample was diluted into 50 mM HEPES buffer, pH 7.0, containing 0.8 mM dithiothreitol, 200 mM KCl, 10 mM $MgCl_2$, and 350 μM [1-^{14}C]IPP (2 μCi/μmol) and assayed for IPP isomerase activity by the acidlability procedure.[13] The results are shown in Table 1. The average of five independent determinations gave 0.96 ± 0.14 atoms of zinc per molecule of IPP isomerase. The average specific activity of these samples was 0.39 ± 0.04 μmol·min^{-1}·mg^{-1}.

Scheme 1. Mechanism for Isomerization of IPP and DMAPP

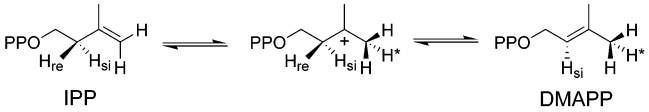

Table 1. Specific Activity and Zinc Content for Type I IPPIsomerase

sample	sp. act. [μmol/min mg]	predicted Zn [μg/mL]	actual Zn [μg/mL]	Zn:enzyme ratio
1	0.35	0.23	0.178	0.774
2	0.44	0.38	0.424	1.116
3	0.43	0.69	0.543	0.787
4	0.37	0.96	0.935	0.974
5	0.34	114	132	1.158

In a second set of experiments, the cells were disrupted in 5 mM phosphate buffer containing 10 μM $MnCl_2$ and 10 μM $ZnCl_2$, and the enzyme was chromatographed on DE52-cellulose using the same buffer. A center fraction gave IPP isomerase that was >95% pure. The sample was desalted over Sephadex G-25 and gently stirred over presoaked Chelex. Samples were taken after 30 min and 24 h, passed over Sephadex G-25, and analyzed for protein content and metal ions. Mn^{2+} levels were at background. The sample contained 0.89 atoms of Zn^{2+}/mol of enzyme after 30 min, and 0.84 atoms/mol after 24 h. Thus, IPP isomerase contains an atom of zinc when purified in metal-free buffers or in buffers containing equal concentrations of Zn^{2+} and Mn^{2+}. The enzyme is fully active in the absence of Mn^{2+} as long as Mg^{2+} is present in the buffer.

IPP isomerase was dialyzed against 1 L of TB and 1 L of TB containing 0.5 mM *o*-phenanthroline. Portions of the solution containing enzyme were removed at $t = 0$, 1, 2, and 6 h. A portion of the sample was diluted into the HEPES buffer and assayed for isomerase activity. A second portion was submitted for metal ion analysis by atomic absorption. The results are shown in Figure 1.

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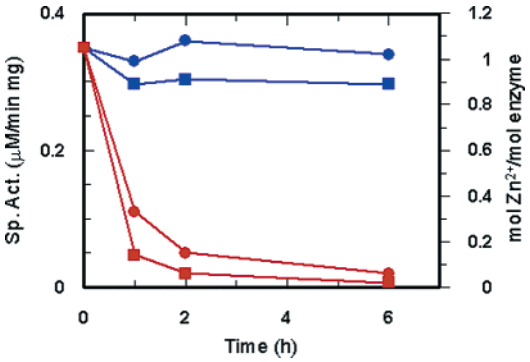

Figure 1. Time-dependent inactivation of IPP isomerase. Specific activity (circles) and zinc content (squares) after dialysis with (red) and without (blue) *o*-phenanthroline in the buffer.

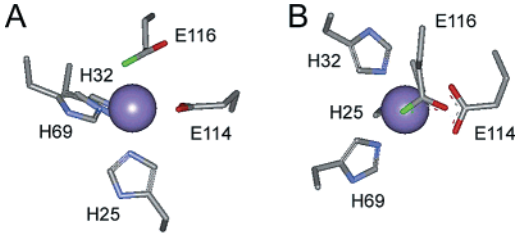

Figure 2. X-ray structure of the Zn^{2+} binding site in type I IPP isomerase. The green oxygen in E116 is coordinated to Zn^{2+}.

Those samples dialyzed against TB retained isomerase activity and zinc, while those dialyzed against TB containing *o*-phenanthroline lost both activity and zinc in a parallel time-dependent manner. Thus, type I IPP isomerase required an atom of Zn^{2+} for activity.

The crystal structure of type I IPP isomerase indicates that the heavier metal is in a six-coordinate binding pocket. As shown in Figure 2, H25, H32, H69, and E116 (green) each provides a single coordination site, while both oxygens of the carboxylate moiety of E114 coordinate to the metal. Interestingly, if one views the bidentate interaction of E114 as a point charge centered at the carboxylate carbon, the ligands around the metal form a slightly distorted trigonal bipyramid with H25 and E116 in the axial locations and E114, H32, H69, and E114 in the equatorial plane. The metal is also important for stabilizing the tertiary structure of IPP isomerase. In a structure of the metal-free protein, the first 30 amino acids, including H25, are disordered, and the imidazole side chain in H32 is rotated away from the three remaining ligands in the metal binding site.

Zinc is found in a large number of proteins, where it fulfills a variety of structural and catalytic roles.[14] Structural zinc sites typically have four protein ligands in a tetrahedral array. Catalytic sites are typically four- or five-coordinate with the ligands in distorted tetrahedral or trigonal biypramidal geometries. In most cases, zinc serves to activate hydroxyl or thiol moieties as nucleophiles. Common examples are the activation of water by carbonic anhydrase[15] and carboxypeptidase,[15] or the activation of cysteine thiols by protein farnesyl and protein geranylgeranyl transferases.[16] In zinc-containing alcohol dehydrogenases[14] and aldolases,[17] the metal is thought to be a Lewis acid catalyst that acts on oxygen atoms in the substrate.

The role of zinc in type I IPP isomerase appears to be different. Although Zn^{2+} stabilizes the structure of IPP isomerase, X-ray structures of enzyme−inhibitor complexes suggest a catalytic role as well.[11] The mechanism for type I IPP isomerase requires an active-site acid sufficiently powerful to protonate an unactivated carbon−carbon double bond. At this point the source of the proton is unclear. E116 is an essential active-site residue that interacts directly with Zn^{2+} through one of its carboxyl oxygens. The other oxygen points toward the IPP binding region and forms a hydrogen bond with ammonium hydrogen in the transition-state analogue. This "transition-state" structure is consistent with a mechanism where E116 provides the proton and, because of its location in the inner coordination sphere of Zn^{2+}, has a substantially lower pK_a than normal. However, if E116 is the acid that protonates the carbon−carbon double bond in IPP, it is not clear how the carboxyl state is maintained prior to catalysis. The same oxygen of E116 is also hydrogen-bonded to the hydroxyl group in Y104, and a more complex network may be involved in the protonation step. C67 is positioned to facilitate removal of a proton from the opposite side of the tertiary carbocation to complete an antarafacial isomerization. This scenario is consistent with the enzyme-bound product formed when IPP isomerase is irreversibly inactivated with the epoxy analogue of IPP. An X-ray structure of the inhibited protein shows that the epoxide ring has been opened to give a hydroxyl group at C(4) and the sulfhydryl moiety in C67 is covalently attached to C(3) of the inhibitor by a thioether linkage.

In summary, type I IPP isomerase contains an atom of zinc. The metal is located in an unusual six-coordinate pocket and may facilitate protonation of the unactivated carbon−carbon double bond in IPP. The sulfhydryl moiety C67, perhaps in the thiolate form, is in position to remove a proton from the resulting tertiary carbocation to complete the reaction.

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