Isopentenyl-Diphosphate Isomerase: Irreversible Inhibition by 3-Methyl-3,4-epoxybutyl Diphosphate†

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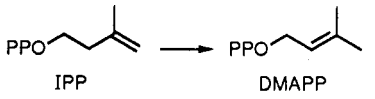
ABSTRACT: Isopentenyl-diphosphate:dimethylallyl-diphosphate isomerase (EC 5.3.3.2) catalyzes the 1,3-allylic rearrangement of the homoallylic substrate isopentenyl diphosphate (IPP) to its allylic isomer, dimethylallyl diphosphate (DMAPP). Incubation of yeast IPP isomerase with 3-methyl-3,4-epoxybutyl diphosphate (EIPP) resulted in a time-dependent first-order loss of activity characteristic of an active-site-directed irreversible process, where $k_2 = 0.63 \pm 0.10$ min^{-1} and $K_I = 0.37 \pm 0.11$ μM. A 1:1 covalent E–I complex was formed upon incubation with [1-^{14}C]EIPP. The inhibited enzyme was treated with trypsin to give two radioactive fragments, which were purified by reversed-phase HPLC on a C_{18} column. The modified amino acid in each fragment was identified as C139 by sequencing the radiolabeled peptides. Incubation of IPP isomerase with [2,4,5-$^{13}C_3$]EIPP gave a ^{13}C-labeled E–I complex. A ^{1}H–^{13}C heteronuclear multiquantum correlation spectrum had strong cross-peaks at 1.2/28 and 2.9/48 ppm, which we assigned to the labeled methyl group and C(4) methylene, respectively, of the inhibitor. In addition, a weak signal at 2.17/42 ppm may be from the C(2) methylene. Comparison of these chemical shifts with those of a synthetic adduct isolated from treatment of EIPP with cysteine indicates C139 attacks C(4) of EIPP to generate a thioether linkage between the enzyme and the inhibitor.

Isopentenyl-diphosphate:dimethylallyl-diphosphate isomerase (EC 5.3.3.2) catalyzes the interconversion of isopentenyl diphosphate (IPP)[1] and dimethylallyl diphosphate (DMAPP).



IPP DMAPP

This is an essential step early in the isoprenoid biosynthetic pathway which provides the potent electrophile DMAPP required for the subsequent prenyl transfer reaction. The pathway is ubiquitous, and over 20 000 naturally occurring isoprenoid metabolites have been identified. End products of the pathway include such important classes of compounds as sterols (Brown & Goldstein, 1979), carotenoids (Britton, 1989), ubiquinones (Bentley et al., 1987), and dolichols (Hemming, 1981). In addition, it was recently discovered that prenylation is an essential posttranslational modification for many proteins that play key roles in membrane structure (Khosravi-Far et al., 1991), signal transduction across membranes (Linder et al., 1991), and transport between organelles (Kinsella & Maltese, 1991).

IPP isomerase catalyzes the interconversion of IPP and DMAPP by a stereoselective antarafacial [1.3] transposition of hydrogen (Cornforth et al., 1966; Cornforth & Popjak, 1969; Clifford et al., 1971). Several lines of evidence point to an electrophilic protonation/deprotonation mechanism for the isomerization of IPP to DMAPP by IPP isomerase from *Saccharomyces cerevisiae* and from *Claviceps purpurea*. These include proton-exchange measurements (Street et al., 1990); decreased reactivities for fluorinated analogs of IPP (Muehlbacher & Poulter, 1988a) and DMAPP (Reardon & Abeles, 1986); potent noncovalent inhibition by ammonium analogs of the putative carbocationic intermediate in the rearrangement (Reardon & Abeles, 1985, 1986; Muehlbacher & Poulter, 1985, 1988a); and irreversible inhibition by epoxy analogs of IPP and DMAPP designed to be mechanism-based inhibitors (Muehlbacher & Poulter, 1988a).

The stereochemistry of the reaction is consistent with an active-site architecture consisting of two active-site residues located on opposite faces of the allyl moiety in IPP to assist with introduction and removal of protons. Recently, *IDI*1, the gene for IPP isomerase in *S. cerevisiae*, was characterized (Anderson et al., 1989), and the yeast enzyme was overproduced in *E. coli* (Street & Poulter, 1990). An active-site nucleophile, the thiol group in C139, was identified by covalent modification of the recombinant enzyme with radiolabeled 3-fluoromethyl-3-butenyl diphosphate by an S_N2 displacement of fluorine (Street & Poulter, 1990; Poulter et al., 1988). We now report work with 3-methyl-3,4-epoxybutyl diphosphate (EIPP) which also labels C139 and permits us to deduce the structure of the modified amino acid.

MATERIALS AND METHODS

Materials. Dowex AG 50W-X8 cation-exchange resin (H^+ form, 100–200 mesh) was purchased from Bio-Rad. The resin was washed in sequence with concentrated HCl, water to pH 7, 30% NH_4OH or 20% nBu_4NOH, and water to pH 7 prior to use in ion exchanges requiring Dowex (NH_4^+ form) or Dowex (nBu_4N^+ form). Reagent-grade acetonitrile was dried over anhydrous $MgSO_4$ and distilled from P_2O_5. Unlabeled EIPP was prepared according to the procedure of Muehlbacher and Poulter (1988). [1-^{14}C]Isopentenyl diphosphate (36.7 μCi/μmol) was purchased from Amersham. [2,4,5-$^{13}C_3$]IPP

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[1] Abbreviations: BSA, bovine serum albumin; DMAPP, dimethylallyl diphosphate; DTT, dithiothreitol; E–I, enzyme–inhibitor; EIPP, epoxyisopentenyl diphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HMQC, heteronuclear multiquantum correlation; HPLC, high-performance liquid chromatography; IPP, isopentenyl diphosphate; NMR, nuclear magnetic resonance; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; TMS, tetramethylsilane; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)-aminomethane; TSP, sodium trimethylsilylpropionate; UV, ultraviolet.

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was available from another study in our laboratory. All other chemicals were purchased from Aldrich or Sigma.

IPP isomerase was purified according to the procedure of Street and Poulter (1990) from freshly transformed cultures of *E. coli* strain JM101/pIPS241. Enzyme activity was measured by the acid-lability procedure using [1-^{14}C]IPP (2 μCi/μmol) prepared by dilution of commercially available material with unlabeled IPP. TPCK-treated trypsin and calf intestinal phosphatase were obtained from U.S. Biochemicals. Dialysis was performed in Spectrapor dialysis bags (MW cutoff 6000–8000 K). Protein solutions were concentrated in an Amicon Centricon-10 stirred ultrafiltration cell (MW cut off 10 000 K) by centrifugation at 4000*g*. Radioactivity was measured in Opti-fluor scintillation media (Packard Instrument Co.). Peptide sequences were provided by Dr. Bob Schackmann, Utah Regional Cancer Center Protein/DNA Core Facility.

NMR Measurements. NMR spectra were obtained in $CDCl_3$ or D_2O (Cambridge Isotopes). ^{1}H and ^{13}C chemical shifts are reported in parts per million downfield from either TMS or TSP, and ^{31}P shifts, in parts per million downfield from external phosphoric acid. ^{13}C NMR spectra of the enzyme–inhibitor complex were acquired at 125.7 MHz and referenced to external sodium trimethylsilylpropionate (TSP). The spectra window was 30 kHz with a total acquisition time of 1.08 s between pulses. Full proton decoupling was accomplished by Waltz-16 decoupling (Shaka et al., 1983) with 1 W of power centered at 3.0 ppm in the proton spectrum. The data were apodized with 5 Hz line-broadening and zero-filled to 64K complex points.

A ^{1}H–^{13}C HMQC spectrum of the inhibited enzyme was acquired at 11.75 T using a standard pulse sequence (Bax et al., 1983) while acquiring 2048 complex points covering a spectral window of 8000 Hz in the T_2 dimension with a relaxation delay of 1 s between pulses. Four thousand hypercomplex T_1 increments (States et al., 1982) were acquired covering a sweep width of 30 kHz. Broad-band ^{13}C decoupling was used during the acquisition. The T_2 FIDs were apodized with a 90° shifted squared sinebell function to 2048 points prior to Fourier transform. The T_1 dimension was apodized with a similar function to 4096 points and zero filled to 8 K points prior to transformation.

Synthesis of [2,4,5-$^{13}C_3$]-3-Methyl-3,4-epoxybutyl Diphosphate ([2,4,5-$^{13}C_3$]EIPP). [2,4,5-^{13}C$_3$]IPP (3.3 mg, 11 μmol) in 0.5 mL of water was loaded onto a 0.5 × 7 cm column of Dowex (nBu_4N^+ form) and eluted with 5 mL of water. After lyophilization, the residue was dissolved in 60 μL of $CHCl_3$, and 6.3 mg of *m*-chloroperbenzoic acid (33 μmol) in 60 μL of $CHCl_3$ was added. After 105 min, $CHCl_3$ was removed with a gentle stream of nitrogen, and the residue was dissolved in 0.5 mL of 25 mM NH_4HCO_3. The mixture was loaded onto a Dowex column (0.5 cm i.d. × 7 cm, NH_4^+ form) and eluted with 2.5 mL of 25 mM NH_4HCO_3. The solution was lyophilized to give a solid containing [2,4,5-^{13}C$_3$]EIPP *m*-chlorobenzoate, and some residual nBu_4NHCO_3. NMR resonances for the epoxide were resolved from those of the other compounds in the mixture. Comparisons of peak intensities for *m*-chlorobenzoate and EIPP indicated the epoxide was obtained in >80% yield and no IPP remained: ^{1}H NMR (300 MHz, D_2O/ND_4OD) for [2,4,5-^{13}C$_3$]EIPP 1.24 (3 H, br d, $J_{H,C}$ = 127 Hz, H at methyl), 1.78 [2 H, br d, $J_{H,C}$ = 149 Hz, H at C(2)], 2.65 [1 H, br d, $J_{H,C}$ = 175 Hz, H at C(4)], 2.74 (1 H, br d, $J_{H,C}$ = 175 Hz, H at C(4)], 3.90 [2 H, br m, H at C(1)]; ^{13}C NMR (125.7 MHz, D_2O/ND_4OD) 22.34 [*q*, $J_{C,H}$ = 127 Hz, C(5)], 38.95 [t, $J_{C,H}$ = 149 Hz, C(2)], 57.41 [t, $J_{C,H}$ = 175 Hz, C(4)]; ^{31}P NMR (121 MHz, D_2O/ND_4OD) −9.71 (1 P, d, $J_{P,P}$ = 19.7 Hz), −10.75 (1 P, d, $J_{P,P}$ = 19.7 Hz).

Synthesis of [1-^{14}C]-3-Methyl-3,4-epoxybutyl Diphosphate ([1-^{14}C]EIPP). A 700-μL solution of tris(ammonium) [1-^{14}C]IPP (6.4 μmol, 36.7 μCi/μmol) was converted to the tris(tetra-*n*-butylammonium) salt by passage through a 0.5 cm × 7 cm column of Dowex (nBu_4N^+ form). The column was eluted with 5 mL of water, and the eluant was lyophilized. The residue was dissolved in 1 mL of dry $CHCl_3$ and transferred to a 1.5-mL vial. The volume of the solution was reduced to 100 μL with a stream of nitrogen before addition of 6.7 mg of *m*-chloroperbenzoic acid (39 μmol). After stirring for 2 h at room temperature, 3.3 mg of Na_2CO_3 was added, followed 5 min later by 20 μL of dimethyl sulfide. Solvent was evaporated with a gentle stream of nitrogen and then in high vacuum (4 mTorr) for 6 h at room temperature. The residue was dissolved in 90 μL of 5 mM NH_4HCO_3, pH 7.0, and stored at −60 °C.

Synthesis of L-(S)-3-Methyl-(1,3-dihydroxy-4-butyl)cysteine. To 100 mg of L-cysteine (0.82 mmol) in 5 mL of liquid NH_3 was added 81 mg (0.82 mmol) of 3-methyl-3,4-epoxybutan-1-ol. After 150 min, during which time the NH_3 was allowed to evaporate slowly, 10 mL of acetonitrile was added, and solvent was then removed in vacuo to give 183 mg of a white solid: ^{1}H NMR (300 MHz, D_2O) 1.13 (3 H, s, CH_3), 1.74 [2 H, t, $J_{H,H}$ = 7.5 Hz, H at C(2)], 2.66 [2 H, s, H at C(4)], 2.93 (1 H, dd, $J_{H,H}$ = 7.2 Hz, $J_{H,H}$ = 14.7 Hz, H at Cys C_β), 3.02 (1 H, dd, $J_{H,H}$ = 4.5 Hz, $J_{H,H}$ = 14.7 Hz, H at Cys C_β), 3.58 [2 H, t, $J_{H,H}$ = 7.5 Hz, H at C(1)], 3.77 (1 H, dd, $J_{H,H}$ = 4.5 Hz, $J_{H,H}$ = 7.2 Hz, H at Cys C_α); ^{13}C NMR (75 MHz, D_2O) 27.93 (q, $J_{C,H}$ = 126 Hz, CH_3), 37.21 [t, $J_{C,H}$ = 144 Hz, C(2)], 44.65 (t, $J_{C,H}$ = 127 Hz, Cys C_β), 46.88 [t, $J_{C,H}$ = 142 Hz, C(4)], 56.81 (d, $J_{C,H}$ = 145 Hz, Cys C_α), 60.64 [t, $J_{C,H}$ = 144 Hz, C(1)], 75.35 [s, C(3)], 176.08 (s, carbonyl); HRMS, *m/z* calcd 224.09501, obsd 224.09564.

Inhibition Constants. Incubations were in glass culture tubes containing a total volume of 178 μL of 50 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 200 mM KCl, 0.5 mM DTT, 1 mg/mL BSA, and inhibitor. The tubes were stored on ice, and each sample was equilibrated at 25 °C before the reaction was initiated by addition of 15 ng of enzyme (19 units/mg). The duplicate samples were incubated along with duplicate controls (no added inhibitor) at 25 °C for each concentration of inhibitor. A 20-μL portion was removed at successive time intervals and diluted 10-fold into a solution of 352 μM [1-^{14}C]-IPP (2 μCi/μmol) in the assay buffer. The samples were then incubated at 37 °C for 10 min, quenched by addition of 0.2 mL of 1:4 (v/v) concentrated HCl:methanol, followed by incubation at 37 °C for 10 min. One milliliter of ligroin was added to each tube, and the tubes were vortexed for 15 s. A 0.5-mL portion of the ligroin layer was removed, mixed with 10 mL of Opti-fluor, and counted. Counts were corrected for background radioactivity resulting from a blank sample containing the same volume of buffer and same concentration of [^{14}C]IPP.

Stoichiometry of Inhibition by *[1-^{14}C]-3-Methyl-3,4-epoxybutyl Diphosphate.* IPP isomerase (140 μg, 19.1 units/mg) was added to a glass culture tube containing 0.38 μmol of radiolabeled inhibitor (36.7 μCi/μmol) in assay buffer. The mixture was incubated at 37 °C for 1 h. The solution was transferred to a Centricon-10 microconcentrator by five 400-μL rinses with 25 mM NH_4HCO_3. Unbound radioactivity was removed by concentration/dilution cycles consisting of addition of NH_4HCO_3 and concentration to a volume of

approximately 0.2 mL. A 20-μL portion of the filtrate was counted for residual radioactivity, and the cycle was repeated until the filtrate was free of radioactivity. The concentrated enzyme–inhibitor complex solution was then diluted to 2.0 mL with 25 mM NH_4HCO_3, and the radioactivity in a 20-μL portion was determined.

Affinity Labeling: Determination of the Site of Modification by [1-^{14}C]-3-Methyl-3,4-epoxybutyl Diphosphate. Following the procedure described above, 3.4 mg of IPP isomerase (19 units/mg) was incubated with 3.3 mmol of [1-^{14}C]EIPP (21 μCi/μmol) at 37 °C for 1 h. After 10 min, less than 2% of isomerase activity remained. Unbound inhibitor was removed by dialysis against 10 mM Tris-HCl, pH 8.0. The labeled protein was precipitated by addition of 12 mL of acetone at 0 °C and collected by centrifugation (3000*g*, 15 min). No significant radioactivity was detected in the acetone supernatant. The protein pellet was dissolved in 160 μL of 8 M urea and transferred to a 5-mL plastic incubation tube. To the protein solution was added 30 μL of 1 M Tris-HCl, pH 8.6, 45 μL of 1 mM $CaCl_2$, 45 μL of 2 M diethanolamine containing 1 mM $MgCl_2$, 2 μL of alkaline phosphatase (14 000 units/mL), 8 μL of TPCK-treated trypsin (50 mg/mL, 256 units/mg), and 394 μL of water. The resulting mixture was incubated for 24 h at 25 °C. A 250-μL portion was loaded onto a Vydac C-18 column equilibrated with 0.1% aqueous TFA and eluted with a linear gradient of 0–80% 3:2 (v/v) acetonitrile:0.1% aqueous TFA over a period of 80 min. The radioactivity eluted as a single peak, which was collected and lyophilized. Lyophilized samples from two HPLC runs were combined and rechromatographed on the same column equilibrated with 1:4 buffer A [2:3 (v/v) 10 mM NH_4OAc, pH 6.8:acetonitrile]:buffer B (10 mM NH_4OAc, pH 6.8) and eluted with a linear gradient of 1:4 buffer A:buffer B to 3:2 buffer A:buffer B over 75 min. The radioactive peak was lyophilized and rechromatographed with a linear gradient of 35:65 buffer A:buffer B to 2:3 buffer A:buffer B over 90 min. The radioactivity corresponded to a single UV-absorbing peak. The sample was lyophilized and submitted for peptide sequencing. Samples from each cycle were collected, and the radioactivity in each was determined.

^{13}C NMR Analysis of the Adduct of [2,4,5-$^{13}C_3$]-3-Methyl-3,4-epoxybutyl Diphosphate and IPP Isomerase. A 2-mL sample of IPP isomerase (14 units/mg) containing 10 mg (0.31 μmol) was concentrated to 200 μL in a Centricon-10 microconcentrator at 4 °C, followed by addition of 60 μL (0.30 μmol) of [2,4,5-$^{13}C_3$]EIPP (5 mM in D_2O) and 10 μL of $MgSO_4$, (1.0 M in D_2O). After incubation for 1 h, a portion of the sample was assayed. Less than 2% of the original enzymatic activity remained. The solution was diluted with 2.3 mL of D_2O and concentrated to 200 μL, as previously described. The dilution/concentration cycle was repeated four more times. The concentrated solution containing inactivated IPP isomerase was transferred to an NMR tube. A control sample containing 10 mg of active IPP isomerase without inhibitor was prepared in the same manner.

RESULTS AND DISCUSSION

Inhibition of IPP Isomerase by EIPP. Incubation of yeast IPP isomerase with EIPP resulted in a concentration-dependent first-order inactivation of the enzyme. Rate constants for the inactivation (k_{inact}) were determined from a semilogarithmic plot of residual isomerase activity versus time (see Figure 1). The rate constant for inactivation at saturating levels of EIPP (k_I) and the inhibition constant (K_I) were determined from a replot of k_{inact} versus $[EIPP]^{-1}$

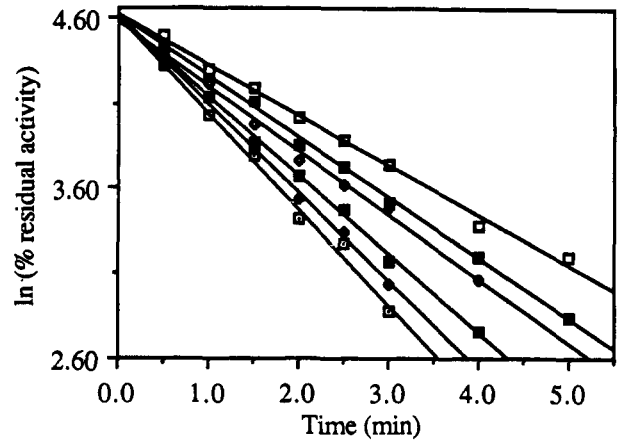


FIGURE 1: Semilogarithmic plot of residual isomerase activity after preincubation with EIPP. Inhibitor concentrations were 0.3, 0.5, 0.7, 1.0, 1.5, and 2.0 μM.

(Muehlbacher & Poulter, 1988). The values for k_I and K_I were 1.1 ± 0.2 × 10^{-2} s^{-1} and 0.37 ± 0.1 μM, respectively. The rate of inhibition was reduced in the presence of IPP in a manner characteristic of protection by substrate. When IPP isomerase was incubated with 2.0 μM EIPP, the half-life to the enzyme increased from 1.2 to 5.7 min in buffer containing 133 μM IPP. These results indicate that inactivation is an active-site-directed process consistent with a mechanism-based inhibition.

The value of K_I for inhibition of yeast isomerase by EIPP is 33-fold higher than for the same inhibitor with the *Claviceps* enzyme (Muehlbacher & Poulter, 1988). Correspondingly, k_I is 4.8-fold higher, resulting in an overall 7-fold decrease in inhibition efficiency (k_I/K_I) for EIPP with yeast isomerase relative to the *Claviceps* enzyme. Part of the difference may arise from inherent differences in binding contributions for the two enzymes since K_m^{IPP} = 2.4 μM for *Claviceps* isomerase (Muehlbacher & Poulter, 1988a), while K_m^{IPP} = 35 μM for the yeast enzyme (Reardon & Abeles, 1986). Reported values of k_{cat} for the two enzymes are also different. The k_{cat} for recombinant yeast isomerase (Street & Poulter, 1990) is seven times greater than k_{cat} for *Claviceps* isomerase (Muehlbacher & Poulter, 1988a). However, we found that yeast isomerase purified from wild-type sources was 5–6-fold less active than recombinant enzyme (Anderson et al., 1989; Street & Poulter, 1990). Thus, the lower k_{cat} reported for *Claviceps* isomerase may simply reflect partial inactivation of the protein during purification.

The stoichiometry of the EIPP–IPP isomerase complex was determined with [^{14}C]EIPP. [^{14}C]EIPP (36.7 μCi/μmol) was synthesized directly from [^{14}C]IPP by epoxidation of the tris(tetra-*n*-butylammonium) salt with *m*-chloroperbenzoic acid in $CHCl_3$. The samples we used for inactivation studies contained ammonium salts of [^{14}C]EIPP and *m*-chlorobenzoic acid, a byproduct of the epoxidation. The concentration of [^{14}C]EIPP was determined by liquid scintillation spectrometry. Control experiments indicated that the benzoate and tetraalkylammonium salts did not interfere with the inactivation reaction at concentrations used during the inhibition experiments. A 140-μg (4.2-nmol) sample of enzyme was inactivated (>99%) with 0.38 μmol of radiolabeled inhibitor. Unbound inhibitor was removed by repeated cycles of dilution/concentration using a Centricon tube. The radioactivity retained by IPP isomerase corresponded to 0.85 mol of bound [^{14}C]EIPP/mol of enzyme.

Identification of C139 as the Active-Site Nucleophile. The site of covalent modification in the EIPP–IPP isomerase

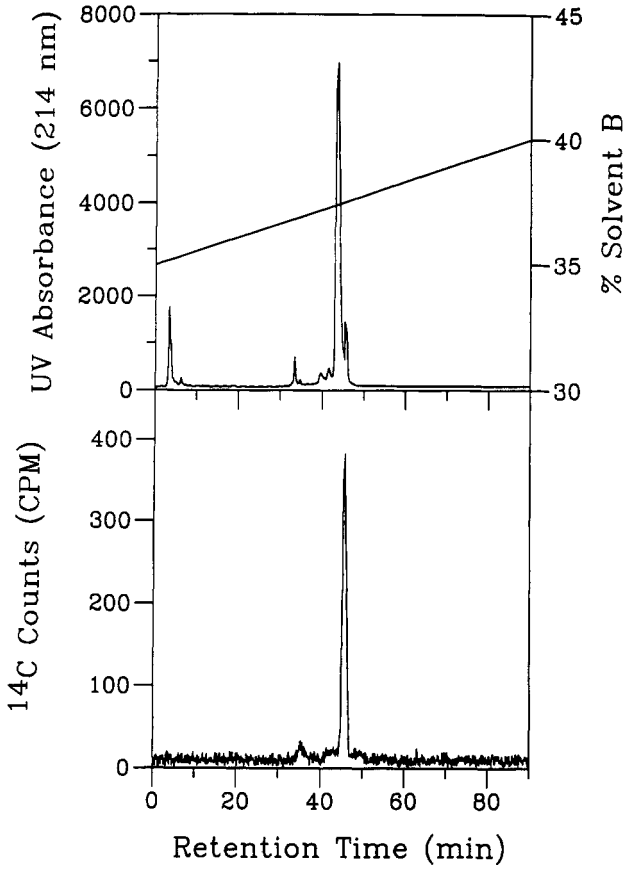
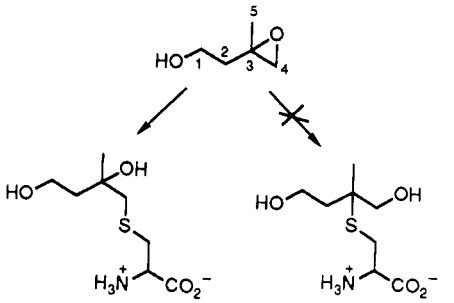


FIGURE 2: HPLC trace of the final chromatography of radioactive peptides from tryptic digests of inhibited IPP isomerase using a linear gradient of buffer A [10 mM $(NH_4)_2SO_4$] and buffer B [3:2 v/v acetonitrile/10 mM $(NH_4)_2SO_4$] as indicated on the plot. The top panel shows the UV trace at 214 nm, and the bottom part shows ^{14}C radioactivity. The radioactivity detector was in series behind the UV detector, giving a slight delay in the ^{14}C peak. The major peaks in both panels correspond to the same elution time.

complex was determined by incubation of enzyme with [^{14}C]-EIPP, followed by proteolysis of the chemically modified protein and sequencing of the radiolabeled fragment. Following the inactivation step, the protein was denatured with 8 M urea, digested with trypsin, and then treated with alkaline phosphatase to remove the diphosphate moiety from the attached EIPP unit. The peptide fragments were separated by reversed-phase HPLC. Three separate chromatographies were required to obtain a major UV-active peak that comigrated with ^{14}C radioactivity; however, the radioactivity eluted consistently as a major peak during all three separations. Absorbance and ^{14}C radioactivity profiles for the final step are shown in Figure 2. In the final separation, the major peak (peptide A) was accompanied by a minor radioactive component with a shorter retention time than the major component (peptide B).

The peptides in fractions corresponding to A and B were collected and sequenced. Both gave clean sequences characteristic of a single component, and both were derivatives of the same region of the enzyme. Peptide A was a 25-mer with the sequence I-T-F-P-D-L-W-T-N-T-X-X-S-H-P-L-X-I-D-D-E-L-G-L-K. Peptide B contained 23 amino acids and was identical to peptide A except it did not contain the C-terminal L-K unit. This sequence exactly matches positions 128–152 in the putative gene product from yeast *IDI*1, which has cysteines at the undetermined positions 138, 139, and 144 in the peptide sequence. Analysis of the radioactivity for individual cycles during sequencing indicated that the inhibitor was attached to C139 in both peptides. This is the same amino acid modified by 3-(fluoromethyl)-3-butenyl diphosphate, although in this case a minor peptide corresponding to 4% of the total bound radioactivity was isolated where alkylation had occurred at C138 (Street & Poulter, 1990).

Structure of the EIPP–C139 Adduct. Alkylation of the sulfhydryl moiety in cysteine could occur at either of the epoxide carbons in EIPP. The structure of the adduct was deduced from HPLC comparisons of the material obtained in cycle 12, which corresponded to C139 during sequencing, with a synthetic sample and from the ^{13}C NMR spectrum of enzyme inhibited with ^{13}C-labeled EIPP. Treatment of 3-methyl-3,4-epoxybutanol with L-cysteine in liquid ammonia gave L-(*S*)-3-methyl(1,3-dihydroxy-4-butyl)cysteine as the sole product. Attempts to generate the regioisomeric 3-butyl



adduct by varying solvent conditions or by acid catalysis were unsuccessful. The modified cysteinyl thioether was converted to its PTH derivative and analyzed under the identical HPLC conditions used during peptide sequencing. The synthetic material had a retention time identical to that for the amino acid released during cycle 12 of the sequence analysis of peptides A and B.

In another set of experiments, IPP isomerase was incubated with [2,4,5-$^{13}C_3$]EIPP, prepared by epoxidation of [2,4,5-$^{13}C_3$]IPP, as described for unlabeled material. ^{13}C NMR and ^{1}H–^{13}C HMQC spectra were obtained for the resulting covalent enzyme–inhibitor complex (see Figures 3 and 4). Part A of Figure 3 shows a ^{1}H-decoupled ^{13}C NMR spectrum of yeast IPP isomerase at 4 °C. Part B of Figure 3 is the region from 35 to 80 ppm. The 10-mg sample of enzyme was then incubated with a 1.5-fold molar excess of [2,4,5-$^{13}C_3$]-EIPP at 37 °C for 1 h, during which time the enzyme lost >99% of its original activity as determined by the standard acid lability assay. Excess inhibitor was removed by repetitive dilution/concentration cycles with buffer made from D_2O. A control sample of IPP isomerase that had not been deactivated with EIPP was treated in an identical manner (part C of Figure 3). Part D of Figure 3 is a spectrum of the enzyme inactivated with labeled EIPP. The only substantial change in the ^{13}C spectra of all three samples of IPP isomerase was in the region near 48 ppm. The spectra of active isomerase before and after the dilution/concentration cycles were similar, whereas, inactivated enzyme gave a new broad resonance centered at 48 ppm.

Figure 4 shows a two-dimensional ^{1}H–^{13}C HMQC spectrum and a one-dimensional projection along the ^{13}C axis for isomerase inhibited with [2,4,5-$^{13}C_3$]EIPP. Strong cross-peaks appear at 1.2/28 and 2.9/48 ppm. The 2.9/48 ppm cross-peak has a carbon chemical shift that corresponds to the new peak seen in the ^{13}C spectrum of inhibited IPP isomerase shown in Figure 3C. The ^{1}H and ^{13}C chemical shifts of this cross-peak are similar to those for the labeled thioether methylene in the 2-methyl-1,3-dihydroxybutyl unit of the synthetic cysteine derivative. On the basis of chemical shifts of the model compound, peaks for the methyl group and the

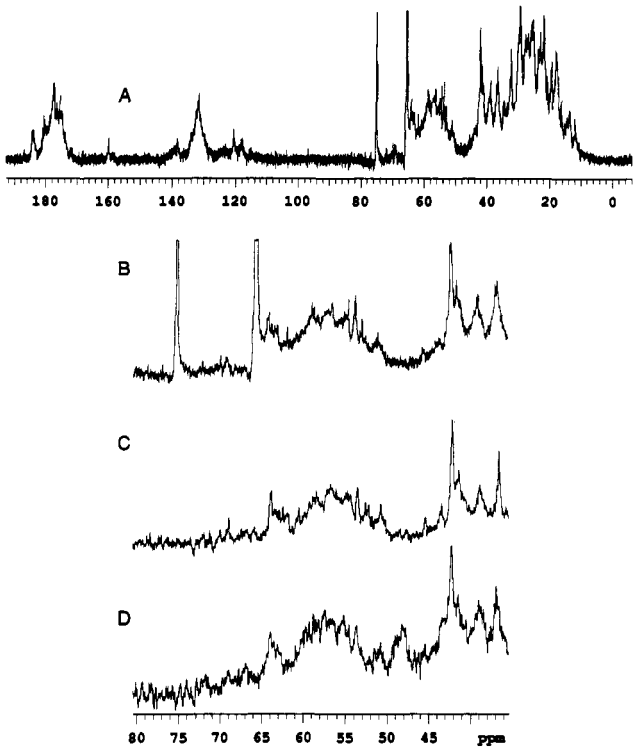


FIGURE 3: ^{13}C NMR spectra of IPP isomerase. (Part A) Full spectrum of IPP isomerase at 4 °C in D_2O buffer containing 3 mM 2-mercaptoethanol. (Part B) Expansion of the region from 35–80 ppm in part A (the peaks at 66 and 75 ppm are from 2-mercaptoethanol). (Part C) Control sample after dialysis against D_2O. (Part D) After inhibition with [2,4,5-$^{13}C_3$]EIPP and dialysis against D_2O.

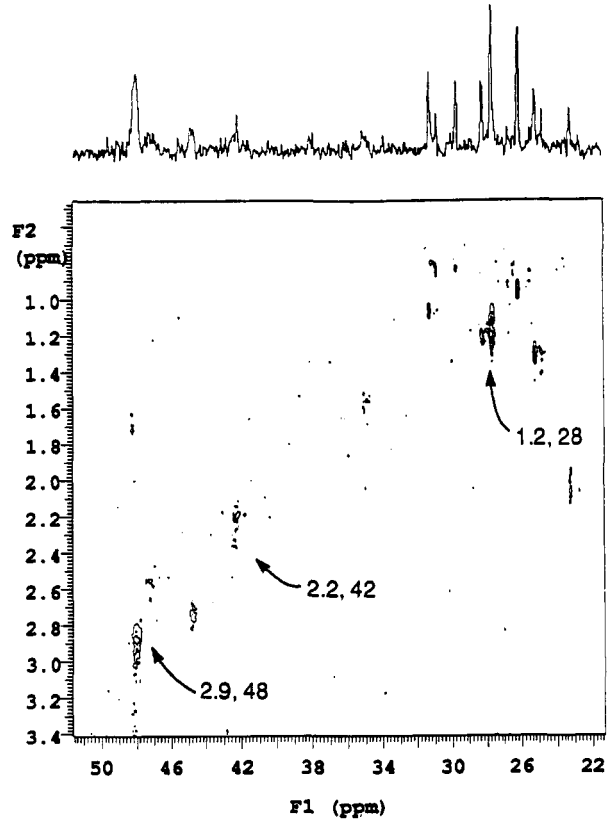
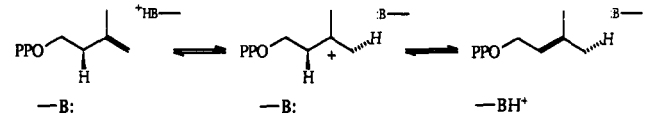


FIGURE 4: Two-dimensional ^1H–^{13}C HMQC spectrum of IPP isomerase inhibited with [2,4,5-$^{13}C_3$]EIPP and a projection along the ^{13}C chemical shift axis.

other labeled methylene group in the E–I complex should occur in congested regions of the ^{13}C spectrum where detection is impossible in a one-dimensional spectrum. We observed a strong cross-peak at 1.2/28 ppm with chemical shifts similar to those of the model and tentatively assigned that resonance to the methyl group. However, several cross-peaks were seen with ^1H shifts between 0.8 and 2.0 ppm and ^{13}C shifts between 23 and 32 ppm, presumably from overlapping resonances for naturally abundant $^1H/^{13}C$ units in the amino acid side chains of the enzyme. It is possible that one of the other cross-peaks near the 1.2/28 ppm resonance is from the methyl group-labeled inhibitor.

Scheme I: "Two-Base" Protonation/Deprotonation Mechanism for Anatarfacial Isomerization of IPP



On the basis of the NMR spectra of the model thioether, we would expect a third cross-peak in the vicinity of 1.7/37 ppm for the other labeled methylene. There are no strong signals in this region, and the closest weak resonance that might arise from the other labeled methylene is at 2.15/42 ppm. We have no explanation why the signal for the third labeled carbon is so weak relative to the other two resonances.

The NMR experiments with [2,4,5-$^{13}C_3$]EIPP are sufficient to establish the regiochemistry of the alkylation reaction. The cross-peak at 2.9/48 ppm in the HMQC spectrum has ^1H and ^{13}C chemical shifts similar to the values of 2.7 and 46 ppm we measured for the C(4) methylene unit attached to sulfur in the 1,3-dihydroxy-3-methylbutyl moiety of our synthetic cysteine thioether derivative. One would anticipate a cross-peak with a ^1H chemical shift between 3 and 4 ppm and a ^{13}C shift below 60 ppm if the C(4) methylene were attached to an oxygen. The absence of a peak in the one- and two-dimensional spectra in this region indicates that the principal product from inactivation of isomerase by EIPP results from attachment of cysteine to the primary epoxy carbon in the inhibitor.

Mechanism of Inhibition. EIPP was designed to take advantage of the protonation/deprotonation sequence shown in Scheme I (Reardon & Abeles, 1986; Muehlbacher & Poulter, 1988a). We reasoned that an active-site acid capable of protonating the double bond in IPP should also protonate the epoxide oxygen in EIPP, thereby activating the moiety toward nucleophilic attack. Since acid-catalyzed opening of an epoxide ring normally proceeds with attachment of the anionic partner to the more substituted carbon, the regiochemistry of our C139–EIPP adduct suggests a nucleophilic process. However, the conversion of IPP to DMAPP by a protonation/deprotonation mechanism requires that C(3) of the carbocationic species be unavailable for reaction with C139 to prevent formation of an unreactive thioether adduct. Since the regioselectivity for acid-catalyzed opening of an epoxide is not particularly high (Muehlbacher & Poulter, 1988b), a similar topological constraint for EIPP coupled with the presence of a good leaving group at C(4) to capture an active-site nucleophile normally involved in the elimination step is sufficient to explain the structure of the adduct within the context of a proton-catalyzed epoxide opening. This scenario is supported by pH–rate profiles for isomerization of IPP and inactivation by EIPP. Both V_{max} and k_I are maximal between pH 6 and pH 7 and decrease sharply above pH 8 (Muehlbacher & Poulter, 1988a). If the sulfhydryl moiety in C139 attacked an unactivated epoxide, one might expect that the rate of alkylation would remain high or perhaps increase, as pH increased from 8 to 9.

The antarafacial stereochemistry for the interconversion of IPP and DMAPP requires two active-site residues to promote the protonation and deprotonation events: one to serve as an active-site acid and the other as a base. Thus far, two active-site cysteines have been identified as sites for covalent modification by IPP analogs. 3-(Fluoromethyl)-3-butenyl diphosphate alkylates C139 and, to a minor extent, C138 (Street & Poulter, 1990) by an S_N2 mechanism (Poulter et al., 1988). PCR-mediated site-directed mutagenesis of C139 created C139A and C139V mutants that were inactive, indicating that the residue is an essential component of the catalytic machinery of IPP isomerase (Street et al., 1991). We originally surmised that only one of the enantiomers of EIPP might react with isomerase. However, a 1.5-fold molar excess of EIPP inactivated the enzyme in the experiments to determine the stoichiometry and to deduce the structure of the adduct by NMR. If the reaction was highly enantioselective, at least 10% of the original activity should remain, based on a 0.25-fold molar deficiency of either enantiomer of EIPP and a preparation of enzyme whose stoichiometry of inactivation was 0.85 mol of inhibitor/mol of enzyme. Our results suggest that C139 is alkylated by both enantiomers of EIPP. The apparent inability of IPP isomerase to distinguish between (*R*)- and (*S*)-EIPP is consistent with recent NMR experiments which show that the active site of the enzyme accommodates at least three different conformers of the normal substrate (Street et al., 1990).

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