Identification of Cys139 and Glu207 As Catalytically Important Groups in the Active Site of Isopentenyl Diphosphate:Dimethylallyl Diphosphate Isomerase†

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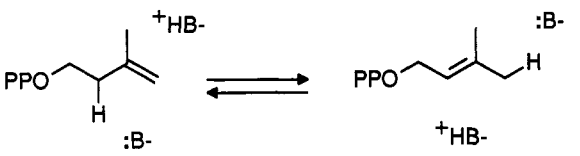
ABSTRACT: Isopentenyl diphosphate:dimethylallyl diphosphate isomerase (EC 5.3.3.2) catalyzes the antarafacial [1.3] allylic rearrangement of isopentenyl diphosphate (IPP) to its electrophilic allylic isomer dimethylallyl diphosphate (DMAPP). Active-site thiols at C138 and C139 were recently identified by covalent modification using active-site-directed irreversible inhibitors [Street, I. P., & Poulter, C. D. (1990) *Biochemistry 29*, 7531–7538; Lu, X. J., Christensen, D. J., & Poulter, C. D. (1992) *Biochemistry 31*, 9955–9960]. Kinetic studies were conducted with site-directed mutants of IPP isomerase (IPPIase) to evaluate the roles of these amino acids. C138S and C138V mutants were active catalysts with V/K values only 10-fold lower than that of wild-type IPPIase. In contrast, the C139S mutant was a poor catalyst, and the C139A and C139V mutants were inactive. Treatment of the C139S mutant with 3-(fluoromethyl)-3-butenyl diphosphate, an electrophilic active-site-directed irreversible inhibitor, resulted in inactivation of the enzyme by covalent modification of E207. The E207Q and E207V mutants were inactive, suggesting a role for the E207 carboxylate moiety in catalysis.

Isopentenyl diphosphate:dimethylallyl diphosphate isomerase (EC 5.3.3.2) catalyzes a crucial "activation" step in isoprenoid biosynthesis by converting isopentenyl diphosphate (IPP)[1] to its highly electrophilic isomer dimethylallyl diphosphate (DMAPP). These two five-carbon building blocks are used to construct the 23 000 isoprenoid compounds found in nature. IPP isomerase (IPPIase) 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). The enzyme has been purified from *Saccharomyces cerevisiae* (Reardon & Abeles, 1986; Street & Poulter, 1990) and *Claviceps purpurea* (Muehlbacher & Poulter, 1988) and subjected to a variety of studies. Several lines of evidence point toward the protonation–deprotonation mechanism shown in Scheme 1 for the isomerization reaction. These include proton-exchange measurements (Street et al., 1990), decreased reactivities for fluorinated analogs of IPP (Muehlbacher & Poulter, 1988) and DMAPP (Reardon & Abeles, 1986), potent noncovalent inhibition by ammonium analogs of the putative carbocationic intermediate (Reardon & Abeles, 1985, 1986; Muehlbacher & Poulter, 1985, 1988), and irreversible inhibition by mechanism-based inhibitors containing epoxide moieties (Muehlbacher & Poulter, 1988; Lu et al., 1992).

The antarafacial stereochemistry of the isomerization is consistent with an active site containing two residues located on opposite faces of the allyl moiety in IPP to assist with addition and elimination of protons. *IDI1*, the gene for IPPIase in *S. cerevisiae*, was recently characterized (Anderson et al., 1989), and an overproducing strain of *E. coli* containing *IDI1* was constructed (Street & Poulter, 1990). A thiol group at C139 was identified as a catalytic-site residue in the recombinant enzyme by covalent modification with radioactive 3-fluoro-3-butenyl diphosphate (FIPP), an affinity label for IPP (Street & Poulter, 1990; Poulter et al., 1988). Replacement of C139 with alanine or valine by site-directed mutagenesis gave inactive enzyme (Street et al., 1991).

Scheme 1



Although the antarafacial stereochemistry of the isomerization suggested that IPPIase has two nucleophilic groups in its catalytic site, efforts to locate a second nucleophile were unsuccessful. These included extensive studies with active-site-directed irreversible inhibitors containing an epoxide moiety (Lu et al., 1992) and a careful examination of the peptides labeled by FIPP (Street & Poulter, 1990). Only when IPPIase was inhibited with FIPP was a small amount (ca. 4%) of the radioactivity found at C138. The epoxides did not label this position. We now report studies with new mutants of IPPIase that uncovered an essential glutamate at position 207 and demonstrated that C138 is not required for catalysis.

MATERIALS AND METHODS

Materials and General Procedures. [1-^{14}C]IPP was purchased from Amersham Corp. FIPP, [4-^{3}H]FIPP (47 μCi/μmol), 2-(dimethylamino)-1-ethyl diphosphate (NPP), [2′-^{3}H]NPP (241 μCi/μmol), and 3,4-epoxy-1-butenyl diphosphate (EBPP) were prepared previously (Muehlbacher & Poulter, 1988). Phenyl Superose HR 10/10 was obtained from Pharmacia LKB Biotechnology Inc.; TPCK-treated trypsin and calf intestinal phosphatase were obtained from U.S. Biochemicals. All restriction enzymes and T4 DNA ligase

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[1] Abbreviations: DMAPP, dimethylallyl diphosphate; DTT, dithiothreitol; EBPP, epoxybutyl diphosphate; FIPP, 3-(fluoromethyl)-3-butenyl diphosphate; HPLC, high-performance liquid chromatography; IPP, isopentenyl diphosphate; IPPIase, isopentenyl diphosphate isomerase; NPP, (*N*,*N*-dimethylamino)ethyl diphosphate; PMSF, phenylmethanesulfonyl fluoride; BME, β-mercaptoethanol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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were purchased from New England Biolabs. Polymerase chain reaction (PCR) was performed with the Gene Amp kit (U.S. Biochemicals). DNA sequencing reactions were performed using the dideoxy chain termination method (Sanger et al., 1977) with Sequenase II (U.S. Biochemicals). Double-stranded templates were used and were prepared according to instructions from the supplier. Plasmid DNA was purified using the Qiagen Hi-purity plasmid kit (Qiagen Inc.). Radioactivity was measured in Optifluor scintillation medium (Packard Instrument Co.) using a Packard Tricarb Model 4530 liquid scintillation spectrometer. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with the discontinuous buffer system of Laemmli (1970), and gels were stained with Coomassie brilliant blue R. Protein concentrations were determined by the method of Bradford (Scopes, 1982) using bovine serum albumin (BSA) as a standard.

Strains, Media, and Transformations. *Escherichia coli* strain DH5α (Bethesda Research Labs) was used for all plasmid manipulations. *E. coli* strain JM101 (Yanisch-Perron, 1985) was used for expression of wild-type and mutant IPPIase. Competent *E. coli* cells were prepared, stored, and transformed by established procedures (Maniatis et al., 1982). For synthesis of IPPIase, cultures of the JM101 transformants were grown on a supplemented M9 minimal medium (M9+CAGM) containing the following: M9 salts and trace elements (Maniatis et al., 1982), casamino acids (1%, w/v), glucose (0.26%, w/v), $MgSO_4$ (0.3 g/L), $CaCl_2$ (0.004 g/L), thiamine hydrochloride (0.0054 g/L), and $FeCl_2$ (0.0054 g/L).

Site-Directed Mutagenesis. Site-directed mutagenesis was performed using counter PCR (Street et al., 1991). The IPPIase expression vector pIPS241 (Street & Poulter, 1990) was used as the template for the PCR. Mutations were introduced at positions 138 and 139 with the following primers (mismatched bases are shown in bold): C139S, 5′-AAC ACA TGC **TCT** TCT CAT CCA CTA-3′ (this primer contained a *Bsp*MI restriction site); C138S, 5′-AAC ACA **TCG** TGC TCT CAT CCA CTA TGT AAT-3′; C138V, 5′-AAC ACA **GTG** TGC TCT CAT CCA CTA TGT ATT-3′ (the latter two primers contained a *Dra*III restriction site). For both sets of mutations, extension of the complementary strand was initiated from the oligonucleotide 5′-AGT CCA AAG ATC AGG GAA AGT TAT T-3′. The following primers were used to introduce mutations at residue 207 and to introduce a unique *Cla*I site: E207Q, 5′-GAA CAT **CAG** ATC GAT TAC ATC CTA TTT TAT-3′; E207V, 5′-GAA CAT **GTT** ATC GAT TAC ATC CTA TTT TAT-3′. The 30-mer 5′-ACC CCA TGG TTC ATT GCT TGG TGC CAT GTA-3′ was used to initiate extension of the complementary strand for the Glu207 mutants.

The PCR mixture contained the following: 100 ng of pIPS241 (Street & Poulter, 1990), 200 μM dNTP, 1 μg of the mutagenic primer, 1 μg of the reverse primer, 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin, and 5 units of *Taq* polymerase in a final volume of 100 μL. This mixture was taken through 15 cycles of the following: denaturation (94 °C, 1.5 min), annealing (40 °C, 2 min), and extension (72 °C, 12 min). After the PCR reaction was complete, the four dNTPs were added to a final concentration of 250 μM each, followed by the Klenow fragment of *E. coli* DNA polymerase (20 units), and the mixture was incubated at 37 °C for 30 min. The DNA product was extracted with phenol, precipitated by ethanol, and subjected to electrophoresis on a 0.8% agarose gel. The 3.4-kb band was isolated from the gel, phosphorylated at the 5′-ends by treatment with 20 units of polynucleotide kinase, and ligated with 20 units of T4 DNA ligase (4–12 h, 22 °C). The ligation mixture was used to transform *E. coli* strain DH5α. Transformants were screened for the correctly mutated IPPIase gene in three stages. Initial screening was done by restriction analysis of DNA minipreps to locate the new restriction site introduced on the mutagenic PCR primer. Then DNA from colonies giving the predicted restriction fragment lengths was used to transform *E. coli* strain JM101, and the transformants were used to produce mutant IPPIase. Fifteen-milliliter cultures of the JM101 transformants were grown in M9+CAGM (containing 50 μg/mL ampicillin) for 8–10 h at 37 °C. The cells were harvested by centrifugation and resuspended in a buffer containing 100 mM potassium phosphate (pH 7.0), 10 mM β-mercaptoethanol (BME), and 1 mM phenylmethanesulfonyl fluoride (PMSF). The cells were disrupted by sonication while being cooled on ice, and the crude extract was clarified by centrifugation (12 000*g*, 12 min). The cell-free extract was assayed for IPPIase activity (see below), and aliquots were subjected to SDS–PAGE. Transformants that produced a protein of the correct molecular mass were selected, and the DNA sequence in the region of the mutation was determined. A single mutant possessing the correct sequence around the mutation site was selected, and the entire coding region of the IPPIase gene was sequenced.

Purification of Recombinant IPPIase. M9+CAGM (330 mL) containing ampicillin (50 μg/mL) was inoculated with 6 mL of a stationary-phase culture of JM101/pIPS241, and the culture was allowed to grow at 37 °C for 8 h. The cells were harvested by centrifugation (7000*g*, 15 min) and stored frozen at −70 °C until needed. Approximately 2 g of cell paste (wet cell weight) was resuspended in 10 mL of buffer containing 100 mM KOAc (pH 5.0), 10 mM BME, and 1 mM PMSF. The cells were disrupted by sonication while being cooled in ice, and the cellular debris was removed by centrifugation (20000*g*, 30 min). To the clarified extract was added an equivalent volume of saturated $(NH_4)_2SO_4$ solution (pH 5.0). The addition was completed over approximately 30 min while the extract was stirred in an ice bath. The $(NH_4)_2SO_4$ precipitate was removed by centrifugation (20000*g*, 30 min), and the supernatant was loaded onto a Phenyl Superose column (HR 10/10) equilibrated with 1.5 M $(NH_4)_2SO_4$, 100 mM KOAc (pH 5.0), and 1 mM dithiothreitol (DTT). The column was washed with starting buffer until the absorbance of the eluate had returned to the baseline, and IPPIase was eluted by a decreasing linear gradient from 1.5 to 0 M $(NH_4)_2SO_4$ (100 mM KOAc, pH 5.0, and 1 mM DTT) at a flow rate of 0.8 mL/min. The total volume of the gradient was 94 mL. IPPIase eluted approximately halfway through the gradient. Fractions containing the enzyme were pooled and concentrated (Centriprep-10, Amicon Corp.). The concentrated enzyme was mixed with an equivalent volume of glycerol and stored at −20 °C.

Assay Procedure. IPPIase assays were performed using the acid lability procedure (Satterwhite, 1985). The assay buffer contained the following: 50 mM HEPES (pH 7.0), 200 mM KCl, 10 mM $MgCl_2$, 1 mg/mL BSA, 0.5 mM DTT, and 350 μM [1-^{14}C]IPP (2 μCi/μmol). The reaction was initiated by addition of enzyme, and incubation was for 10 min at 37 °C. The reaction was quenched by addition of 0.2 mL of HCl/methanol (1:4, v/v), and incubation was continued at 37 °C for 10 min. Acid-labile material was extracted by vortexing with 1 mL of ligroine (bp 90–120 °C), and a 0.5-mL sample of the ligroine layer was removed for liquid scintillation spectrometry.

Kinetic Measurements. The kinetic parameters K_M and V were determined from initial rates at seven different substrate concentrations. Assay mixtures contained assay buffer, 0.01–1 mM [1-^{14}C]IPP (2 μCi/μmol), and IPPIase. The reaction was initiated by addition of enzyme, and incubation was continued for 10 min at 37 °C. The mixtures were quenched, and formation of DMAPP was measured by using the acid lability procedure described above. The kinetic parameters were calculated by fitting initial velocities to the nonlinear form of the Michaelis–Menten equation using a nonlinear regression analysis (Enzfitter, Elsevier Biosoft).

The pH dependence of the kinetic constants for the C139S mutant was measured as follows. Assay mixtures contained 50 mM KOAc (pH 4.5–5.5), MES (pH 5.37–6.65), HEPES (pH 6.62–7.87), Tris (pH 7.72–8.75), or CHES (pH 8.79–9.82) buffer in solutions containing 200 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 1 mg/mL BSA, 50–700 μM [1-^{14}C]-IPP, and 160 μg of C139S IPPIase. The reaction was initiated by addition of enzyme, and incubation was continued for 10 min at 37 °C. The assay mixtures were quenched and were analyzed by the acid lability procedure. The pH-rate profiles were analyzed by the nonlinear regression analysis of Cleland (1979).

The inhibition constant (K_I) was measured as follows. Mixtures contained assay buffer, 50–300 μM [1-^{14}C]IPP (2 μCi/μmol), 5–50 μM NPP, and 200 μg of C139S IPPIase in a total volume of 100 μL. The reaction was initiated by addition of enzyme, and incubation was continued for 10 min at 37 °C. The assay mixtures were quenched, and products were analyzed by the acid lability procedure.

The effect of irreversible inhibitors for IPPIase on the activity of the C139S mutant was determined by incubating 160 μg of C139S IPPIase in assay buffer at 25 °C with 50 μM FIPP, 50 μM EBPP, 1 mM NPP, or 1 mM iodoacetamide. Incubation was continued for 30 min, at which time [1-^{14}C]-IPP was added to the assay buffer to a final concentration of 1 mM. Residual IPPIase activity was determined after a 10-min incubation at 37 °C using the standard acid lability procedure. The amount of inhibition that occurred during incubation with substrate was determined by the same procedure except 1 mM [1-^{14}C]IPP was added to the buffer containing the inhibitor before addition of enzyme. A parallel set of experiments containing 0.2 μg of wild-type IPPIase was also performed.

Stoichiometry of C139S IPPIase–Inhibitor Complexes. C139S IPPIase (5–15 μg) was incubated in assay buffer (without BSA) at 25 °C for 120 min with an excess of [4-^{3}H]-FIPP (48 μCi/μmol) or [2′-^{3}H]NPP (241 μCi/μmol). The solutions were diluted to a final volume of 1 mL with Tris-HCl (pH 7.4) and transferred to microconcentrators (Centricon-10, Amicon Corp.). The microconcentrators were spun at 5400g until the original sample volume had been reduced to 50–100 μL. The radioactivity of the filtrate was then measured. The concentrate was repeatedly diluted and reconcentrated until the radioactivity in the filtrate had dropped to background levels. The radioactivity of the concentrate was then measured.

Proteolytic Digestion of FIPP-Labeled C139S IPPIase. C139S IPPIase (6.9 mg, 0.21 μmol) was inactivated with [4-^{3}H]FIPP as described above. The labeled protein (19 μCi/μmol) was precipitated by addition of an equivalent volume of 20% trichloroacetic acid (TCA) and collected by centrifugation (12000g, 15 min). The pellet was washed with 10% TCA and then dissolved in 1 mL of buffer containing 0.1 M Tris-HCl (pH 8.5) and 4 M urea. The remaining unbound inhibitor was removed by an overnight dialysis against 4 L of 10 mM Tris-HCl (pH 8.5). The labeled protein was lyophilized, redissolved in 500 μL of water, and digested with 1/10 (w/w) TPCK-treated trypsin (251 units/mg). The digestion mixture also contained 50 mM Tris-HCl (pH 8.5), 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 2 M urea, and 65 units of calf intestinal phosphatase. The digestion was continued for 20 h at room temperature.

Separation of Tryptic Peptides by Reversed-Phase High-Performance Liquid Chromatography (HPLC). The tryptic peptides from labeled C139S IPPIase were separated on a Vydac C-18 peptide column (0.46 × 25 cm) using a Waters 501 HPLC system equipped with a Waters Associates Model 441 absorbance detector. The eluate was monitored at 214 nm and by detection of radioactivity using a Flo-One\β radioactive flow detector. Initial separation of the peptide mixture was accomplished using the following conditions: buffer A, 0.1% TFA; buffer B, acetonitrile/water (3:2, v/v) containing 0.1% TFA. The column was equilibrated with buffer A. Peptides were eluted using a linear gradient starting at 100% buffer A and decreasing to 0% buffer A over 80 min. A flow rate of 1 mL/min was used, and fractions of 1 mL were collected. Approximately 80% of the total radioactivity in the sample eluted as a single peak, and fractions belonging to this region were combined and lyophilized. Final purification of the labeled peptide was accomplished using a solvent system consisting of buffer C (10 mM NH_4OAc, pH 6.8) and buffer D (acetonitrile/buffer C (3:2, v/v)). The combined fractions were rechromatographed with a shallow, linear gradient starting at 65% buffer C and decreasing to 50% buffer C over 60 min (flow rate = 1 mL/min). Fractions containing radioactivity were combined and lyophilized. The resulting radiolabeled peptide was chromatographically pure and submitted for sequence analysis by automated Edman degradation on an Applied Biosystems Model 477A protein sequencer. The peptide (14.6 nmol, 16 μCi/μmol) was judged from the results of the amino acid sequence analysis to be greater than 98% pure.

RESULTS

Site-Directed Mutagenesis. Counter PCR is a useful method for performing site-directed mutagenesis (Hemsley et al., 1989; Street et al., 1991). The initial amplification is directed by two primers located "back to back" on a circular template. One of the primers contains the mismatches which generate the site-directed mutation, while the other primer directs extension of the complementary strand. Unlike the more conventional methods of PCR-mediated site-directed mutagenesis (Higuchi et al., 1988; Kadowaki et al., 1989; Valette et al., 1989), counter PCR does not rely on the presence of conveniently located restriction sites.

The mutagenic primers for IPPIase contained base changes necessary to alter the amino acid codon and to introduce a silent mutation that created a new restriction site. This allowed positive clones to be easily identified by analysis of restriction fragments. The presence of the desired mutation was determined by DNA sequencing.

The IPPIase expression vector pIPS241 (Street & Poulter, 1990) was amplified efficiently by PCR. Starting with 100 ng of template, 15 cycles of PCR was sufficient to produce enough DNA for the subsequent steps. After gel purification of the 3.4-kb band and processing as described by Hemsley et al. (1989), the ligation mixture was used to transform *E. coli* strain DH5α. A good yield of colonies was obtained, and approximately 70% of the transformants gave positive re-

Table 1: Kinetic Parameters for Mutant IPPIases

IPPIase	V (μmol min^{-1} mg^{-1})	K_M (μM)	V/K_M
wild type	20	43	0.47
C138S	5.3	87	0.06
C138V	4.6	93	0.05
C139S	1.2×10^{-3}	181	6.7×10^{-6}
C139A	$<10^{-4}$ [a]	nd[b]	
C139V	$<10^{-4}$ [a]	nd[b]	
E207Q	$<10^{-4}$ [a]	nd[b]	
E207V	$<10^{-4}$ [a]	nd[b]	

[a] Minimum level of activity detectable by the standard assay procedure. [b] nd = not determined.

Table 2: Inactivation of C139S IPPIase

inhibitor	% inactivation[a] C139S	wild type
none	0	0
FIPP (50 μM)	100	100
EPBB (50 μM)	11	100
NPP (1 mM)	100[b]	100
iodoacetamide (1 mM)	2	36

[a] Percentage of inactivation after a 30-min incubation in the presence of the inhibitor. [b] Inhibition is not time-dependent.

striction fragment patterns. Between 50 and 90% of the positive clones synthesized a protein of the expected size, and 100% of the double-positive clones possessed the correct DNA sequence at the mutation site. In some experiments the mutational frequency dropped dramatically. This was attributed to incomplete phosphorylation before ligation and was remedied by phosphorylation of the primers prior to the PCR reaction.

Mutation of Cys138 and Cys139. In a recent publication, Street and Poulter (1990) showed that FIPP was a potent active-site-directed inhibitor of yeast IPPIase. The primary site of attachment in the covalent enzyme–inhibitor complex was Cys139. Approximately 4% of FIPP was attached at the preceding residue, Cys138. These two cysteine residues were the initial targets for site-directed mutagenesis. Two substitutions were made at position 138, the sterically conservative substitution Cys to Ser (C138S) and substitution of the cysteine with a bulky hydrophobic residue, Cys to Val (C138V). Both C138S and C138V IPPIase had considerable activity and were purified to greater than 90% homogeneity by a simple procedure consisting of $(NH_4)_2SO_4$ precipitation followed by chromatography on Phenyl Superose. The kinetic parameters for the C138S and C138V mutants are shown in Table 1. In both cases, the values of V were reduced approximately 4-fold relative to that of the wild type, while K_M increased approximately 2-fold. In contrast, substitutions at Cys139 had a dramatic effect on enzymatic activity. For the C139S mutant, V decreased 10 000-fold, and K_M increased 4-fold. The corresponding C139A and C139V mutants were inactive within the detection limits of the assay.

Since the level of activity observed for C139S IPPIase was very low relative to that of the wild-type enzyme, it was necessary to establish that the residual activity seen in samples of C139S IPPIase was due specifically to the mutant enzyme and not a contaminant from the *E. coli* host. This was accomplished by studying the effects of irreversible IPPIase inhibitors on the C139S mutant, and the results are summarized in Table 2. Incubation of C139S IPPIase with 50 μM FIPP for 30 min resulted in complete inactivation of the enzyme. The stoichiometry of the FIPP–C139S IPPIase complex was determined with radiolabeled FIPP. Samples of C139S IPPIase were inactivated with an excess of [4-^{3}H]-FIPP. Unbound inhibitor was removed by repeated washing of an ultrafiltration membrane, and the radioactivity remaining in the enzyme corresponded to 0.8 mol of FIPP per mole of C139S IPPIase. The observation that FIPP completely inhibited isomerase activity in C139S IPPIase and formed a stoichiometric complex with this enzyme suggests that the enzymatic activity in these samples was due specifically to the mutant enzyme.

Several differences were noted between C139S IPPIase and the wild-type enzyme with respect to their susceptibility to different inhibitors. Wild-type IPPIase was readily inhibited by 3,4-epoxybutyl diphosphate (EBPP) (Muehlbacher & Poulter, 1988), while this compound had little effect on the activity of the C139S mutant. The thiol-selective reagent iodoacetamide inactivated wild-type IPPIase by alkylation of an active-site thiol group (Reardon & Abeles, 1986; Agranoff et al., 1960), presumably Cys139 (Street & Poulter, 1990). In our hands, wild-type isomerase lost 36% of its activity when incubated with 1 mM iodoacetamide at pH 7.0. This value is in good agreement with a half-life of approximately 45 min predicted for similar conditions from the kinetic data in the previous study by Reardon and Abeles (1986). However, iodoacetamide had little effect on the activity of the C139S mutant, which lacks a thiol moiety at that position. (*N*,*N*-Dimethylamino)ethyl diphosphate (NPP) is an ammonium analog of the tertiary carbocationic intermediate proposed for the interconversion of IPP and DMAPP and is a powerful inhibitor of IPPIase (Muehlbacher & Poulter, 1985, 1988; Reardon & Abeles, 1985, 1986). NPP binds irreversibly, but noncovalently, to IPPIase with $K_D \leq 10^{-11}$ M and is released from the enzyme only upon denaturation of the protein, or at high pH where the inhibitor is no longer protonated. Initial results suggested that NPP was also a good inhibitor of C139S IPPIase. However, further investigation showed that inhibition was not time-dependent and that NPP was removed from the protein upon dialysis under nondenaturing conditions. Analysis of initial velocities at different NPP concentrations showed that the compound was a competitive inhibitor of synthesis of DMAPP from IPP by C139S IPPIase with K_I = 42 μM.

A plot of V/K versus pH for C139S IPPIase gave a broad bell-shaped curve like that reported for wild-type enzyme (data not shown), except the acid and alkaline arms of the curve were both shifted to lower pH and corresponded to functional groups with pK of 4.9 and 8.3. These values are shifted by 0.6 and 1.1 pK units, respectively, from those reported for the acid and alkaline limbs of V/K profiles for wild-type enzyme (Reardon & Abeles, 1986). The validity of these shifts was verified by measuring V_{max} versus pH for wild-type yeast IPPIase in our series of buffers from pH 7.8 to 10.0. The pH-rate profile predicted an ionizable group with a pK at 9.4, in excellent agreement with the previous report (Reardon & Abeles, 1986).

Site of Covalent Attachment in the FIPP–C139S IPPIase Complex. To determine the site of covalent attachment in the FIPP–C139S IPPIase complex, samples of the enzyme were inactivated with radiolabeled FIPP. The labeled protein was denatured and digested with trypsin, and the diphosphate moiety was removed from the inhibitor by treatment of the mixture of peptides with alkaline phosphatase. The tryptic peptides were purified by HPLC. A single radioactive peptide was obtained, and the material was sequenced by automated Edman degradation. The sequence that was obtained, M-A-P-S-N-E-P-W-G-E-H-E-I-D-Y-I-L-F-Y-K, did not follow a

trypsin cleavage site in IPPIase and was probably a result of chymotryptic or nonenzymatic cleavage. The possibility that a new trypsin cleavage site had been inadvertently created during site-directed mutagenesis was excluded since the entire coding region of the C139S mutant was sequenced. The isolated peptide exactly matches positions 196–219 of the amino acid sequence of IPPIase. Radioactivity was released in cycle 12 of the sequencing run, corresponding to the position for Glu207 in the amino acid sequence of IPPIase. Thus, Glu207 was identified as the site of covalent attachment in the FIPP–C139S IPPIase complex.

Site-Directed Mutagenesis of Glu207. The identification of Glu207 as the site of covalent attachment in the FIPP–C139S IPPIase complex provided a new target for site-directed mutagenesis. The coding region for IPPIase was altered by counter PCR to create E207Q and E207V mutants. The mutant recombinant enzymes were purified from *E. coli* hosts, as described for wild-type IPPIase and the C139 mutants. The glutamate mutants showed no activity in the isomerase assay at levels where approximately 10^{-5} of wild-type activity would have been detected (see Table 1).

DISCUSSION

The *E. coli* expression system for IPPIase described by Street and Poulter (1990) was ideal for mutagenesis studies. The extremely low levels of native IPPIase activity in *E. coli* (Sherman et al., 1989) coupled with the high levels of recombinant enzyme produced in the host made it possible to measure low levels of activity from the mutant enzyme in crude cell-free extracts in preliminary screens. The Cys residues at positions 138 and 139 were chosen as the initial targets for site-directed mutagenesis, on the basis of results from a previous labeling study (Street & Poulter, 1990) which utilized FIPP as an active-site-directed irreversible inhibitor (Muehlbacher & Poulter, 1988). The principal site of covalent attachment in the FIPP–IPPIase complex was Cys139, although a small amount of inhibitor was attached to Cys138. Since Street and Poulter (1990) had previously found that all of the cysteines in wild-type yeast IPPIase were in the reduced form, the thiol moieties in C138 and C139 were both potential candidates for essential catalytic-site residues. Substitution of Cys138 by Ser or Val did not have a substantial effect on the activity of IPPIase. In both cases the catalytic efficiency (V/K_M) was reduced by approximately 10-fold relative to that of wild-type enzyme, suggesting that Cys138 does not play an essential role in catalysis or in maintaining tertiary structure. In contrast, V/K_M for the C139S mutant was reduced to 10^{-5} of that for wild-type IPPIase, mostly as the result of a large decrease in V. These results suggest that Cys139 has an important role in stabilizing the transition state. Inhibition studies with the C139S mutant support this conclusion. NPP is a slow-binding, irreversible inhibitor of wild-type IPPIase ($K_D \sim 10^{-11}$ M; Reardon & Abeles, 1986; Muehlbacher & Poulter, 1988) that mimics the putative carbocationic intermediate in the isomerization reaction. Studies of C139S IPPIase with NPP showed a reversible competitive inhibition kinetic profile with $K_I = 42$ μM. A comparison of K_D for NPP with wild-type IPPIase with K_I for the C139S mutant indicates that replacement of the sulfhydryl by a hydroxyl at that position results in approximately a 9 kcal/mol decrease in binding energy for the transition-state analog. It is difficult to account for the large decrease in binding energy upon replacing the -SH moiety with -OH by differences in hydrogen bonding, and further studies are needed to resolve the factors responsible for the change seen in the C139S mutant.

Reardon and Abeles (1986) also presented evidence for involvement of a thiol group during catalysis. They found that yeast IPPIase was inactivated by iodoacetamide and that the rate of inactivation depended on a single ionizable group of pK 9.3. The pK value determined for this thiol group was similar to the pK value of 9.4 which they measured for the alkaline limb of a pH-rate profile for the enzyme. Their kinetic data suggest that the catalytically important thiol is protonated under normal physiological conditions.

Substitution of the thiol group in Cys139 by a hydrogen (C139A) or a methyl group (C139V) abolished catalytic activity. The reduced activity of the C139S mutant might result from the decreased acidity of the serine hydroxyl relative to that of the cysteine thiol. However, the pH-rate profile of C139S IPPIase suggests a more subtle explanation. V/K_M for the C139S mutant depended on ionizable groups at pK_a = 4.9 and 8.4, whereas values of 5.5 and 9.4 were determined for the wild-type enzyme (Reardon & Abeles, 1986). If Cys139 was acting as a simple acid catalyst, one might have expected a larger difference between wild-type and mutant enzymes.

Several attempts to locate a second active-site nucleophile required for an antarafacial isomerization by treating wild-type IPPIase with a variety of active-site-directed irreversible inhibitors (Lu et al., 1992) failed. However, incubation of the C139S mutant with FIPP resulted in covalent modification of E207. Presumably, substitution of the thiol moiety at position 139 by a hydroxyl reduced the nucleophilicity of the side-chain nucleophile and allowed the E207 carboxylate to compete for the electrophilic inhibitor. However, it is interesting to note that C138 was not alkylated in the C139S mutant in contrast to the results we obtained for wild-type enzyme (Street & Poulter, 1990). Perhaps these differences are due to subtle variations in the conformations of the noncovalent E·I complexes of wild-type and mutant enzymes. The loss of activity in E207Q and E207V mutants suggests that the carboxylate moiety is a crucial part of the catalytic machinery for yeast IPPIase.

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