Tetrahedron Vol. 47, No. 31, pp. 5919-5924, 1991
Printed in Great Britain

0040-4020/91 $3.00+.00

ISOPENTENYL DIPHOSPHATE ISOMERASE. SITE-DIRECTED MUTAGENESIS OF CYS139 USING "COUNTER" PCR AMPLIFICATION OF AN EXPRESSION PLASMID

Ian P. Street, Hazel R. Coffman, and C. Dale Poulter*
Department of Chemistry, University of Utah
Salt Lake City, Utah 84112

(*Received in USA 9 February* 1991)

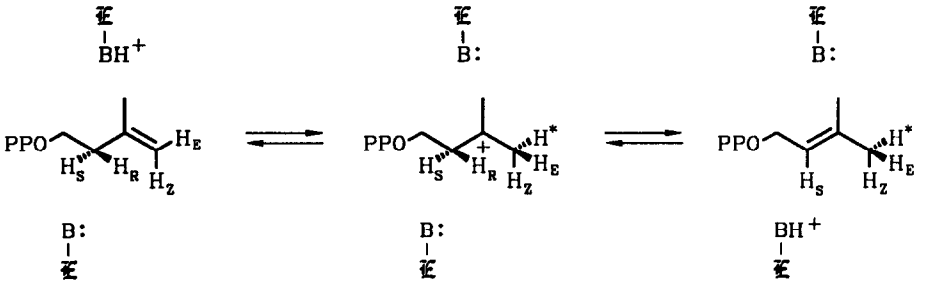
Abstract. Site-directed mutagenesis of Cys139 in yeast isopentenyl diphosphate isomerase was accomplished by "counter" polymerase chain reaction amplification of the gene in expression plasmid pIPS241 using only two DNA primers. One primer complemented a region of the sense strand and contained the mutations. The second primer was an exact complement of the antisense strand. When hybridized to the plasmid template, the 5'-ends of the primers butted against one another, and PCR amplification resulted in synthesis of double strand linear mutant expression plasmid. The linear DNA was circularized by blunt end ligation to yield an expression plasmid that overproduced mutant yeast enzyme in transformed *E. coli* hosts. Two site-directed mutants, C139A and C139V, were both inactive, suggesting that Cys139 is an essential part of the enzyme's catalytic machinery.

INTRODUCTION

The isoprene biosynthetic pathway produces a variety of metabolites that are essential to the host organism. Among the numerous examples found in nature are sterols (structural components of eukaryotic membranes, hormones), carotenes (photoreceptors, coloring agents), ubiquinones (respiratory coenzymes), cytokinins (plant hormones), long chain prenols (sugar carriers in biosynthesis of bacterial cell walls and eukaryotic glycoproteins), and prenylated proteins (signal transduction and membrane scaffolding). Isopentenyl diphosphate isomerase (IPP isomerase) catalyzes the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by an antarafacial [1.3] transposition of hydrogen.[1] This is a required activation step that provides electrophilic DMAPP, the primer for all subsequent prenyltransfer reactions in the pathway. Several lines of evidence, including studies with alternate substrates and transition state/reactive intermediate analogs, indicate that the mechanism of action for the enzyme involves a protonation-deprotonation process as depicted in Scheme 1.[2,3] The antarafacial stereochemistry for proton shuttling suggests the presence of at least two catalytic site residues operating on opposite faces of the double bond.

We recently isolated the gene encoding IPP isomerase from *Saccharomyces cerevisiae*[4] and constructed a heterologous expression system to overproduce the enzyme in *E. coli*.[5] The overproduced enzyme constituted 30-35% of total soluble cellular protein and was indistinguishable from the enzyme purified from yeast. 3-(Fluoromethyl)-3-butenyl diphosphate (FIPP) inactivated IPP isomerase in a time-dependent manner characteristic of an active-site directed irreversible inhibitor to form a 1:1 enzyme-inhibitor complex.[3,5] The primary site of covalent attachment was determined to be Cys139 by inactivation of IPP isomerase with [4-^{3}H]FIPP, digestion of the labeled enzyme with trypsin, purification of the resulting radioactive peptides by reversed-phase HPLC, and Edman degradation to locate the radiolabeled residue.[5] This paper describes site-directed mutagenesis of Cys139 to see if that residue is an essential component of the catalytic machinery of IPP isomerase. The procedure we developed is based on a report by Hemsley et al.[6] for introducing frame shift mutations into the β-galactosidase gene by using two primers located "back-to-back" on the circular plasmid template. For our studies, we added a silent mutation which created a new

Scheme 1. Carbocationic Mechanism for Isomerization of IPP to DMAPP



restriction site used for preliminary screening and then selected transformants that overproduced protein of the proper molecular weight for further study.

EXPERIMENTAL SECTION

Materials and General Procedures. [1-^{14}C]IPP was purchased from Amersham Corp. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Polymerase chain reactions (PCR) were performed with the Gene Amp Kit (U. S. Biochemicals). DNA sequencing reactions were performed using the dideoxy-chain termination method with Sequenase II (U. S. Biochemicals). Double stranded templates were used and were prepared according to the instructions in the sequencing kit. Plasmid DNA was purified using a Qiagen Hi-purity plasmid kit (Qiagen Inc.). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed using the discontinuous buffer system of Laemmli,[7] and gels were stained with coomassie brilliant blue R. Protein concentrations were determined by the method of Bradford[8] 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[9] was used for expression of wild type and mutant IPP isomerase. Competent *E. coli* cells were prepared, stored, and transformed by established procedures.[10] For expression of enzyme, cultures of the JM101 transformants were grown on a supplemented M9 minimal medium (M9+CAGM) containing the following: M9 salts and trace elements,[10] 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 the PCR procedure of Hemsley et al.[6] The IPP isomerase expression vector, pIPS241,[5] was used as the template for the PCR. Mutations were introduced with the following primers (the mismatched bases are shown in bold print): Cys 139 to Ala139 (C139A), 5'-AAC ACC TGC **GCC** TCT CAT CCA CTA TGT ATT-3'; Cys139 to Val 139 (C139V), 5'-AAC ACC TGC **GTT** TCT CAT CCA CTA TGT ATT-3'. These primers contained silent mutations that created a new BspM I restriction site (ACCTGC). For both mutations, extension of the complementary strand was initiated from the oligonucleotide 5'-AGT CCA AAG ATC AGG GAA AGT TAT T-3'. Primers were 5'-phosphorylated before PCR, by treatment with polynucleotide kinase. The PCR mixture contained the following: 50 fmole of pIPS241, 400 μM of each dNTP, 1 μg of each primer, 10 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 denaturation (94 °C, 1.5 min), annealing (40 °C, 2 min) and extension (72 °C, 12 min). After PCR was complete, each dNTP was added to a final concentration

of 250 μM, followed by Klenow fragment (20 units), and the mixture was incubated at 37 °C for 30 min. The DNA was extracted with phenol, precipitated by ethanol, and subjected to electrophoresis on a 0.8% agarose gel. The 3.4 kb band was electroeluted from the gel and ligated with 100 units of T4 DNA ligase (4-12 h, 22 °C). The ligation mixture was used to transform *E. coli* strain DH5α.

Transformants were screened in three stages. Initial screening was done by restriction analysis of miniprepped DNA to locate clones containing the new BspM I restriction site. *E. coli* strain JM101 was then transformed with DNA from colonies giving the correct restriction map, and the transformants were used to express mutant IPP isomerase. Cultures of the transformants were grown in M9+CAGM (15 Ml, 50 μg/Ml ampicillin) for 8-10 h at 37 °C. The cells were harvested by centrifugation and resuspended in buffer containing 100 Mm KH_2PO_4 (pH 7.0), 10 mM 2-mercaptoethanol (BME), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were disrupted by sonication, while being cooled on ice. The suspension was clarified by centrifugation (12 000 x g, 12 min). The cell-free extract was assayed for IPP isomerase activity, and aliquots were subjected to SDS-PAGE. Transformants were selected which produced a protein of the correct molecular mass on the SDS-gels and the DNA sequence in the region of the mutation determined. A single mutant, possessing the correct sequence around the mutation site, was selected, and the entire coding region of the IPP isomerase gene was sequenced.

IPP Isomerase Assays. IPP isomerase assays were performed in duplicate at 37 °C in pH 7.0 in buffer containing 50 mM HEPES, 200 mM KCl, 10 mM $MgCl_2$, 1 mg/mL BSA, 0.5 mM dithiothreitol (DTT), and 350 μM [1-^{14}C]IPP (2 μCi/μmol) using the acid lability protocol.[4,5]

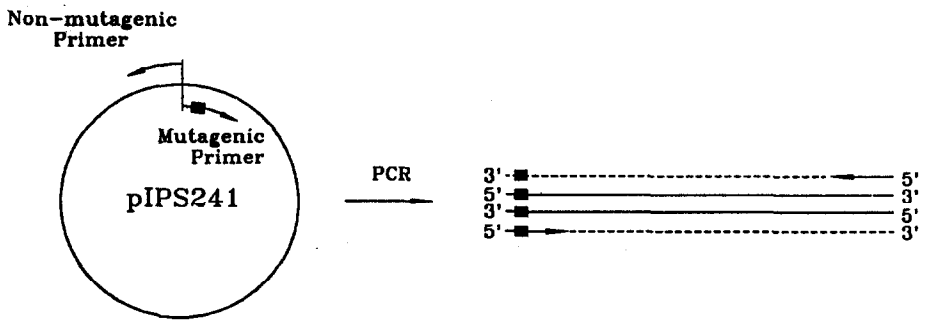
Purification of IPP Isomerase. In a typical run, 330 mL of M9+CAGM containing ampicillin (50 μg/mL) were inoculated (2% v/v) with a stationary-phase culture of a transformant. The culture was incubated in a 2.8 L Fernbach flask for 9 h at 37 °C. The cells were harvested by centrifugation (7,000 g, 15 min). The cell paste was resuspended in buffer containing 100 mM KOAc (pH 5.0), 10 mM BME and 1 mM PMSF to a final density of 2 g/10 mL. The cells were disrupted by sonication while being cooled on ice. The extract was clarified by centrifugation (20,000 g, 30 min), and an equal volume of saturated $(NH_4)_2SO_4$ was added over a period of 30 min. The precipitate was removed by centrifugation (20,000 g, 30 min), and the supernatant was pumped directly 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 DTT. The column was washed with starting buffer until the absorbance of the eluate returned to baseline, and IPP isomerase was eluted with a decreasing linear gradient, 1.5 M to 0 M $(NH_4)_2SO_4$, 100 mM KOAc (pH 5.0), 1 mM DTT, at a flow rate of 0.8 mL/min. The total volume of the gradient was 94 mL. IPP isomerase eluted at approximately 0.75 M $(NH_4)_2SO_4$, and fractions containing the enzyme were pooled and concentrated (Centriprep-10, Amicon Corp.).

RESULTS AND DISCUSSION

Site-Directed Mutagenesis. Site-directed mutagenesis is a powerful technique for demonstrating that specific amino acid residues are critical to enzyme function. Recently, several groups used PCR for site-directed mutagenesis by introducing mismatches, deletions, or insertions into the DNA primers for the extension reaction.[11-13] PCR synthesizes DNA that includes the primers and regions of the template between their 3' ends. Since the regions to be mutated are not normally at the ends of a gene, most procedures for site-directed mutagenesis by PCR use two sets of primers and two separate rounds of PCR. For our work we were attracted to a report by Hemsley et al.[6] They introduced a frame-shift mutation into the gene for β-galactosidase with a single set of primers positioned back-to-back on a circular template. To be successful, the first cycle of PCR must be sufficiently long to traverse around the entire

circular plasmid. Subsequent cycles exactly reproduce a linear version of the original overexpression plasmid containing mutations present in the primers (see Scheme 2). Ligation generates a new circular overexpression

Scheme 2. Site-Directed Mutagenesis by Counter PCR



plasmid containing mutations introduced in the primers. Because 5' → 3' extension in the long direction around the plasmid is opposite to that normally used in PCR, we prefer to call the technique "counter" PCR.[14]

Yeast IPP isomerase is irreversibly inhibited upon S_N2 displacement of the allylic fluoride in FIPP by the sulfhydryl moiety of Cys139 to form a thioether.[5] We decided to see if that residue was essential for catalysis by replacing the sulfhydryl with a hydrogen (C139A mutant) and with methyl groups (C139V mutant). The primers we used for counter PCR site-directed mutagenesis are shown in Scheme 3. The same non-mutagenic primer, which

Scheme 3. Primers Used to Construct C139A and C139V Mutants of IPP Isomerase by Counter PCR Site-Directed Mutagenesis

```
      → 3'-T TAT TGA AAG GGA CTA GAA ACC TGA-5' non-mutagenic primer

5'..GAA AAA ATA ACT TTC CCT GAT CTT TGG ACT AAC ACA TGC TGC TCT CAT CCA CTA TGT ATT GAT ...3'
3'..CTT TTT TAT TGA AAG GGA CTA GAA ACC TGA TTG TGT ACG ACG AGA GTA GGT GAT ACA TAA CTA ...5'

        mutagenic primer (BspM I, C139A) 5'-AAC ACC TGC GCC TCT CAT CCA CTA TGT ATT-3' →
        mutagenic primer (BspM I, C139V) 5'-AAC ACC TGC GTT TCT CAT CCA CTA TGT ATT-3' →
```

was an exact complement to the coding strand and gave 5' → 3' elongation in the counterclockwise direction on pIPS241,was used for both reactions. The mutagenic primers gave elongation in the clockwise direction. That for C139A contained a silent mutation in the codon for Thr137 (ACA → ACC) to introduce a unique BspM I restriction site (ACCTGC) and a *double* mutation in the codon for Cys139 (TGC → GCC) to reduce the probability of reversion. The mutagenic primer for C139V contained the BspM I mutation and a triple mutation in the codon for Cys139 (TGC → GTT). Following synthesis of mutant DNA by PCR, the material was treated with Klenow to remove overhanging 3'-A's sometimes introduced by Taq polymerase. Blunt end ligation of synthetic DNA gave circular expression plasmids for mutant IPP isomerase.

A three-part screening procedure was developed to isolate the desired clones. Minipreps of plasmid from *E. coli* transformants were treated with BspM I and screened for the new site by agarose gel electrophoresis. Positive

colonies were expanded, and proteins in crude cell free extracts were analyzed by SDS-PAGE to screen for overproduction of isomerase-length protein. Finally, the IPP isomerase coding region in selected positive clones was sequenced in the region of the mutation. The entire gene was then sequenced for clones selected for overproduction of C139A and C139V. The results are given in Table 1. In both cases good yields of transformants were obtained.

Table 1. Analysis of Site-Directed Mutation by Counter PCR for C139A and C139V

Mutant	Colonies Picked	BspM I Positive	SDS PAGE Positive	Mutation Sequence Positive
C139A	12	8/12	5/5	3/3
C139V	16	3/16	3/3	2/3

The screening procedure permitted us to rapidly identify clones with the expected mutations that overproduced isomerase. The entire IPP isomerase gene was sequenced for a triply positive clone from each set. The C139A mutant contained no other changes in the isomerase gene, while the C139V had a silent point mutation F187F (TTT → TTC). In some preliminary experiments where 5'-phosphorylation was conducted after gel purification of the PCR product, the mutational frequency dropped precipitously. This was remedied by phosphorylation of the three primers before PCR.

Purification and Kinetic Analysis of C139A and C139V. Our original two-column purification of IPP isomerase from *E. coli* strain JM101/pIPS241[5] was streamlined to a one-column procedure. Following disruption of the cells by sonication and clarification by centrifugation, the supernatant was brought to 50% saturation by $(NH_4)_2SO_4$. Precipitated protein was removed by centrifugation, and the supernatant was loaded directly onto a Phenyl Superose (HR 10/10) column. The column was eluted with a decreasing $(NH_4)_2SO_4$ gradient, and IPP isomerase eluted as a single peak after other adsorbed proteins. SDS PAGE indicated the enzyme was > 95% pure. With this procedure, it was possible to obtain pure IPP isomerase within 20 hours after inoculation of the growth medium. The results of a typical purification are given in Table 2. The C139A and C139V mutants also eluted from phenyl superose after other absorbed proteins as single bands.

Table 2. Purification of IPP Isomerase Overproduced in *E. coli* JM101/pIPS241

purification step	total units (μmol min^{-1})	total protein (mg)	sp act (U mg^{-1})
crude extract	137	32.8	4.2
phenyl superose	68	4.5	15.1

Kinetic analysis of the C139A and C139V mutants indicated both were inactive as catalysts for isomerization of IPP to DMAPP. Based on the specific activity of the wild-type enzyme (sp. act. 15 U mg^{-1}) and the limits of the radioassay for the reaction, we could have easily detected specific activities as low as 1×10^{-4} U mg^{-1}. Thus, replacement of the sulfhydryl moiety at Cys139 with a non-nucleophilic hydrogen atom or with methyl groups, severely

reduced, and may have abolished, catalytic activity. Although the C139A and C139V mutants are inactive, their behavior during chromatography in phenyl superose suggests that the binding affinity of the catalytic site for hydrophobic residues remains intact. We, therefore, think it unlikely that the conservative changes made at position 139 have substantially altered the remaining structural features of the enzyme.

At this point, we don't know the functional role of C139. Reardon and Abeles[2] presented kinetic evidence for involvement of a thiol group in catalysis. They found that yeast IPP isomerase was inactivated by the thiol selective reagent, iodoacetamide, and that the rate of inactivation depended on a single ionizable group of pK 9.3. This value was similar to the pK of 9.4 measured for the alkaline arm of the pH/rate profile for the enzyme. However, a sulfhydryl moiety seems to be a rather weak acid for protonation of an unactivated carbon-carbon double bond in a kinetically competent manner. Additional site-directed mutants of IPP isomerase are being prepared to further elucidate the role of Cys139.

Acknowledgments. This project was supported by NIH grant GM 25521.

REFERENCES

1. Poulter, C. D.; Rilling, H. C. "Biosynthesis of Isoprenoid Compounds", J. W. Porter & S. L. Spurgeon, Eds., Vol. 1, John Wiley & Sons: New York, 1981, pp 209-224.
2. Reardon, J. E.; Abeles, R. H. *Biochemistry* **1986**, *25*, 5609-5616.
3. Muehlbacher, M.; Poulter, C. D. *Biochemistry* **1988**, *27*, 7315-7328.
4. Anderson, M. S.; Muehlbacher, M.; Street, I. P.; Proffitt, J.; Poulter, C. D. *J. Biol. Chem.* **1989**, *264*, 19169-19175.
5. Street, I. P.; Poulter, C. D. *Biochemistry* **1990**, *29*, 7531-7538.
6. Hemsley, A.; Arnheim, N.; Toney, M. D.; Cortopassi, G.; Galas, D. J. *Nucleic Acids Res.* **1989**, *17*, 6545-6551.
7. Laemmli, U. K. *Nature* **1970**, *227*, 680-685.
8. Scopes, R. "Protein Purification. Principles and Practice", Springer-Verlag: Berlin, 1982, p 266.
9. Yanisch-Perron, C.; Vieira, J., Messing, J. *Gene (Amst.)* **1985**, *33*, 103-109.
10. Maniatis, T.; Fritsch, E. F.; Sambrook, J. "Molecular Cloning. A Laboratory Manual", Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1982.
11. Higuchi, R.; Keummel, B.; Saiki, R. K. *Nucleic Acids Res.* **1988**, *16*, 7351-7367.
12. Kadowaki, H.; Kadowaki, T.; Wondisford, F. E.; Taylor, S. I. *Gene* **1989**, *76*, 161-166.
13. Valette, F.; Mege, E.; Reiss, A.; Adensink, M. *Nucleic Acids Res.* **1989**, *17*, 723-733.
14. This term is taken from the "counter" play in football where the running back moves in an opposite direction than anticipated by the defenders based on the flow of the rest of the offensive team.