Isopentenyldiphosphate:Dimethylallyldiphosphate Isomerase: Construction of a High-Level Heterologous Expression System for the Gene from *Saccharomyces cerevisiae* and Identification of an Active-Site Nucleophile†

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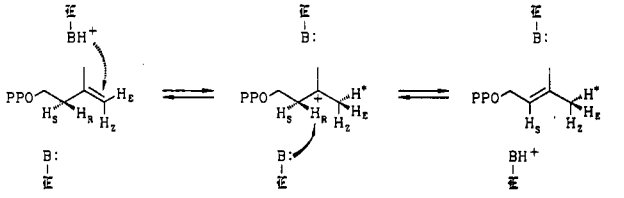
Received March 27, 1990; Revised Manuscript Received May 4, 1990

ABSTRACT: Isopentenyldiphosphate:dimethylallyldiphosphate isomerase (IPP isomerase) is an enzyme in isoprene metabolism which catalyzes the interconversion of the fundamental five-carbon homoallylic and allylic diphosphate building blocks for the pathway. The gene encoding IPP isomerase has recently been isolated from *Saccharomyces cerevisiae* [Anderson, M. S., Muehlbacher, M., Street, I. P., Proffitt, J., & Poulter, C. D. (1989) *J. Biol. Chem. 264*, 19169–19175]. A heterologous expression system was constructed for the gene and used to overexpress IPP isomerase in *Escherichia coli*. In transformants carrying the expression vector, IPP isomerase activity was increased by over 100 000-fold relative to that of the untransformed host strain. The overexpressed enzyme constitutes 30–35% of the total soluble cell protein and can be purified to homogeneity in two steps. Recombinant IPP isomerase was indistinguishable from that purified from yeast. 3-(Fluoromethyl)-3-butenyl diphosphate (FIPP) is a specific active-site-directed inhibitor of IPP isomerase from *Claviceps purpurea* [Muehlbacher, M., & Poulter, C. D. (1988) *Biochemistry 27*, 7315–7328]. Inactivation of yeast IPP isomerase by FIPP was active-site-directed, and inhibition resulted in formation of a stoichiometric enzyme–inhibitor complex. The site of covalent attachment in the enzyme–inhibitor complex was determined by inactivating IPP isomerase with [4-^{3}H]FIPP, followed by digestion of the labeled enzyme with trypsin and purification of the resulting radioactive peptides by reversed-phase high-performance liquid chromatography. The primary site of attachment was Cys-139.

The isoprene biosynthetic pathway is present in some form in all living organisms (Spurgeon & Porter, 1981). A few examples of products formed by this pathway include sterols (structural components of eukaryotic membranes, hormones), carotenes (photoreceptors in visual and photosynthetic systems), glyceryl ethers (archaebacterial membranes), respiratory coenzymes (quinones), insect juvenile hormones, cytokinins (plant hormones), and polyprenol diphosphates (sugar carriers in biosynthesis of bacterial cell wall polysaccharides and eukaryotic glycoproteins). Recent discoveries also suggest an important function for isoprenes in posttranslationally modified protein where isoprene thioethers near the carboxy terminus provide lipophilicity required for membrane association (Rilling et al., 1990; Farnsworth et al., 1990). Two prenylated proteins, Ras and lamin B, play essential roles during cell division (Shafer et al., 1989).

Isopentenyldiphosphate:dimethylallyldiphosphate isomerase (IPP isomerase) fulfills an important role in the isoprenoid biosynthetic pathway. This enzyme catalyzes the interconversion of isopentenyl diphosphate (IPP)[1] and dimethylallyl diphosphate (DMAPP) by an antarafacial [1.3] transposition of hydrogen (Scheme I) (Poulter & Rilling, 1981). DMAPP is a potent electrophile and acts as a primer for all of the subsequent prenyl transfer reactions in the pathway. The stereochemistry of the reaction is consistent with a mechanism involving two bases, one of which is in the conjugate acid form, to assist in the introduction and removal of protons (Clifford et al., 1971). Two limiting possibilities for the initial step consistent with solvent isotope effects are protonation of the double bond to generate a tertiary carbocation and removal of the allylic hydrogen to generate an allylic carbanion

Scheme I: Two-Base Cationic Mechanism for IPP Isomerase



(Agranoff et al., 1960; Shah et al., 1965; Stone et al., 1969). Recently, it was found that the compound 2-(dimethylamino)ethyl diphosphate, an ammonium analogue to the tertiary carbocation proposed for the cationic mechanism, was a powerful inhibitor of IPP isomerase (Muehlbacher & Poulter, 1985, 1988; Reardon & Abeles, 1985, 1986).

Although IPP isomerase activity is ubiquitous, successful purifications of this enzyme have been reported from only three sources: porcine liver (Banthorpe et al., 1977; Bruenger et al., 1986), *Claviceps purpurea* (Bruenger et al., 1986; Muehlbacher & Poulter, 1988), and *Saccharomyces cerevisiae* (Reardon & Abeles, 1986; Anderson et al., 1989). However, due to the difficulties in obtaining IPP isomerase in any reasonable quantity, there is only the most basic structural information available for this enzyme (molecular weight and subunit composition) and virtually nothing known about the enzymatic functional groups which are involved in catalysis.

We have recently reported the isolation and characterization of the gene encoding IPP isomerase from *S. cerevisiae* (An-

† This work was supported by NIH Grant GM 25521 and by the Amoco Corp.
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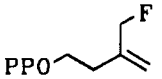
[1] Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FIPP, 3-(fluoromethyl)-3-butenyl diphosphate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; BME, β-mercaptoethanol; KP_i, potassium phosphate; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

0006-2960/90/0429-7531$02.50/0

derson et al., 1989), providing for the first time the amino acid sequence of this enzyme. We now describe the construction of an efficient prokaryotic expression vector for the yeast IPP isomerase gene. The recombinant enzyme can be purified from *Escherichia coli* in two steps with a high overall yield. The ready availability of IPP isomerase allowed us to continue an earlier study on the mechanism of active-site-directed irreversible inhibition of this enzyme by 3-(fluoromethyl)-3-butenyl diphosphate (FIPP). Inactivation of IPP isomerase by



FIPP

this compound was shown to occur by covalent modification of the enzyme (Muehlbacher & Poulter, 1988; Muehlbacher et al., 1989). The results of experiments designed to locate the site of covalent attachment in the inactivated enzyme and thus identify enzymatic groups which could potentially participate in the catalytic mechanism of IPP isomerase are presented.

Materials and Methods

Materials and General Procedures. [1-^{14}C]IPP was purchased from Amersham Corp. FIPP and [4-^{3}H]FIPP (48 μCi/μmol) were prepared previously (Muehlbacher & Poulter, 1988). Microcrystalline DE52 ion exchange cellulose was obtained from Whatman, and octyl-Sepharose was obtained from Pharmacia LKB Biotechnology Inc. Synthetic oligonucleotides were prepared by the Biotechnology Division, Amoco Technology Co. *N*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) treated trypsin and calf intestinal phosphatase were obtained from U.S. Biochemicals. Radioactivity was measured in Optifluor scintillation media (Packard Instrument Co.) with a Packard Tricarb Model 4530 liquid scintillation spectrometer. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of proteins were performed in the discontinuous buffer system of Laemmli (1970) and 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.

Recombinant DNA Methods and Reagents. Large- and small-scale plasmid preparations were performed by standard methods (Maniatis et al., 1982). DNA fragments were purified on 0.8% agarose gels (SeaKem, FMC), and the purified DNA was extracted from the gel matrix with Elutrap (Schleicher & Schuell Inc.). Sequencing was performed by the dideoxy chain termination method of Sanger et al. (1977) using the Sequenase kit (U.S. Biochemicals). Sequencing reactions were performed on double-stranded templates prepared according to instructions from the supplier. Plasmid preparations used for sequencing were purified with the Qiagen Hi-purity plasmid kit (Qiagen Inc.), according to the manufacturer's instructions. All restriction enzymes and T4 DNA ligase were obtained from New England Biolabs or Pharmacia LKB Biotechnology Inc. Polymerase chain reactions (PCR) were conducted with the GeneAmp kit (U.S. Biochemicals). The plasmid pARC133B, which contains the IPP isomerase gene from *S. cerevisiae*, was prepared previously (Anderson et al., 1989). The *E. coli* expression vector pARC306N was obtained from M. Bittner (Biotechnology Division, Amoco Research Corp.).

Strains, Media, and Transformations. E. coli strain DH5α (Bethesda Research Labs) was used for all plasmid manipulations. *E. coli* strain JM101 (Yanisch-Perron et al., 1985) was used for expression of recombinant IPP isomerase. Competent *E. coli* cells were prepared, stored, and transformed

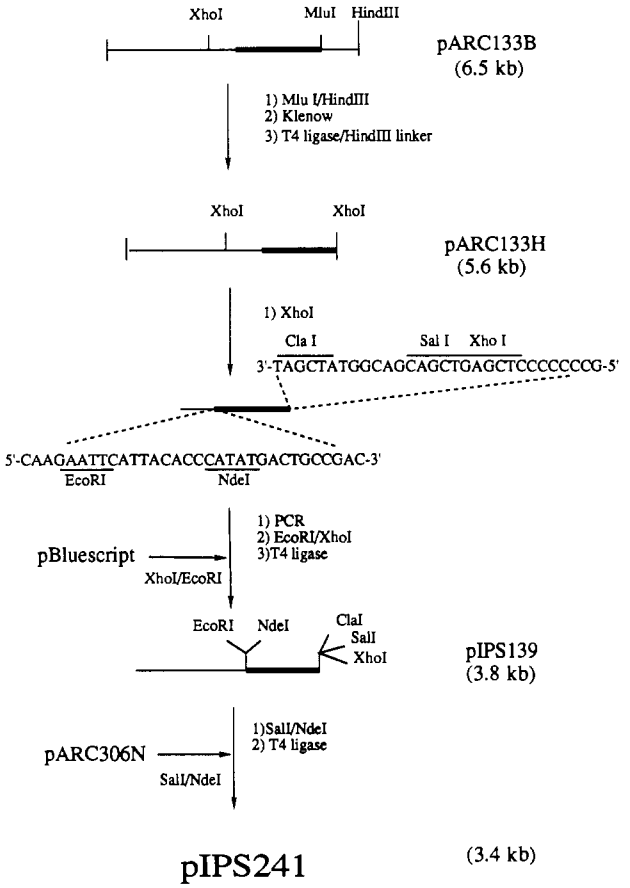


FIGURE 1: Construction of a reading-frame cassette and a prokaryotic expression vector for IPP isomerase. The native yeast terminator was removed from pARC133B as a *MluI*/*Hin*dIII fragment. The resulting plasmid, pARC133H, was digested with *Xho*I, and the 1.75-kb fragment was used as a template for the PCR. The reading-frame cassette was constructed by *Eco*RI/*Xho*I digestion of the PCR fragments followed by ligation into pBluescript SK(+). The bacterial expression vector pIPS241 was constructed by ligating the 0.87-kb *Nde*I/*Sal*I fragment derived from pIPS139 to the 2.52-kb *Nde*I/*Sal*I fragment of pARC306N.

by established procedures (Maniatis et al., 1982). For expression studies, cultures of 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), magnesium sulfate (0.3 g/L), calcium chloride (0.004 g/L), thiamin hydrochloride (0.0054 g/L), and ferrous chloride (0.0054 g/L).

Construction of a Reading-Frame Cassette and a Prokaryotic Expression Vector for the IPP Isomerase Gene. The strategy used in the construction of a reading-frame cassette for the IPP isomerase gene is outlined in Figure 1. The plasmid pARC133B was digested with *Mlu*I and *Hin*dIII and treated with the Klenow fragment of *E. coli* DNA polymerase I. Ligation of the resulting blunt-end fragments in the presence of a synthetic *Hin*dIII linker [d(pCCAAGCTTGG), New England Biolabs] gave a 5.6-kb plasmid pARC133H. Two restriction sites were introduced into the region immediately upstream from the translation initiation codon by PCR-mediated, site-directed mutagenesis. Template DNA for the PCR was prepared from pARC133H by isolation of a 1.75-kb fragment after restriction endonuclease digestion with *Xho*I. The primers used in the PCR are described in Figure 1. The PCR reaction mixture contained the following: 250 ng of template DNA, 1 μg of each primer, 200 μM each of dGTP, dATP, dTTP, and dCTP, and 5 units of *Taq* DNA polym-

erase. This mixture was taken through five cycles with the following temperature changes: denaturation (2 min, 95 °C), annealing (3 min, 37 °C), and extension (2 min, 75 °C). After the fifth cycle, *Taq* polymerase was inactivated by phenol extraction, and the DNA was precipitated with ethanol. The PCR product mixture was digested with *Eco*RI and *Xho*I and then ligated into the *Eco*RI and *Xho*I sites of pBluescript SK(+) (pIPS139, 3.83 kb). The insert was sequenced to ensure that no unwanted mutations had been introduced into the reading frame by the PCR. The 0.87-kb IPP isomerase reading frame was isolated from pIPS139 after *Nde*I/*Sal*I digestion and ligated to the 2.52-kb *Nde*I/*Sal*I fragment of the bacterial expression vector pARC306N. The resulting IPP isomerase expression vector (pIPS241, 3.39 kb) was used to transform *E. coli* strain JM101, and the transformants were used for expression studies and enzyme isolation.

Expression and Induction. Cultures of JM101/pIPS241 were grown on M9 + CAGM containing 50 μg/mL ampicillin at a temperature of 30 or 37 °C with vigorous aeration. Typically, a 30-mL culture was grown in a 500-mL flask and agitated at a rate of 270–300 rpm. The effect of induction by nalidixic acid upon the expression levels of IPP isomerase was determined as follows: 30 mL of M9 + CAGM containing 50 μg/mL ampicillin was inoculated (2% v/v) with a stationary-phase culture of JM101/pIPS241. The culture was allowed to grow to mid log phase (OD_{590} = 1.0–1.4), and nalidixic acid was added to a final concentration of 50 μg/mL. Incubation was continued for a further 2–4 h. The cells were harvested by centrifugation (5000*g*, 15 min), and the cell paste was resuspended in extraction buffer [100 mM potassium phosphate (KP_i), 10 mM β-mercaptoethanol (BME), 1 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.0]. The cells were disrupted by sonication (Bronson sonifier cell disrupter Model 350 equipped with a microprobe), while being cooled in ice–water at a power setting of 2 and a duty cycle setting of 70%. Sonication was carried out for three cycles of 30 s, the extract being allowed to cool between each cycle. The extract was clarified by centrifugation (10000*g*, 20 min), and IPP isomerase activity and total protein concentration were determined as described under Kinetic Measurements.

Purification of Recombinant IPP Isomerase. Three 330-mL portions of M9 + CAGM containing ampicillin (50 μg/mL) were inoculated (2% v/v) with a stationary-phase culture of JM101/pIPS241. Each 330-mL culture was incubated in a 2.8-L Fernbach flask for 9 h at 37 °C. The cells were harvested by centrifugation (5000*g*, 15 min), and the cell paste was resuspended in extraction buffer to a final density of 2 g/10 mL. The cells were disrupted by sonication (0.5-in. disrupter, power setting of 6, 70% duty cycle, 4 × 40 s), while being cooled in an ice bath. The extract was clarified by centrifugation (10000*g*, 20 min), and the total protein concentration and IPP isomerase activity were determined. The clarified extract was then diluted with extraction buffer to a protein concentration of less than 10 mg/mL. The diluted extract was pumped onto a 10 × 5 cm DE52 column (flow rate, 7 mL/min) which had been equilibrated with 100 mM KP_i and 10 mM BME, pH 7.0. The column was eluted with starting buffer until the absorbance of the eluate had returned to the baseline. IPP isomerase was eluted as a single peak with 500 mM KP_i and 10 mM BME, pH 7.0. Fourteen-milliliter fractions were collected. Fractions containing IPP isomerase were combined and concentrated to a final volume of 20 mL with a Minitan-S (Millipore Corp.) tangential flow concentrator (MW cutoff 10000, pump speed = 6, back pressure = 5 psi). The concentrated extract was loaded onto a 2.5 × 25 cm octyl-Sepharose column which had been equilibrated with 500 mM KP_i and 10 mM BME. A flow rate of 1 mL/min was used. IPP isomerase was eluted with the same buffer, and fractions containing the highest specific activity were combined and stored frozen at −70 °C. SDS–PAGE of the purified IPP isomerase indicated that it was greater than 95% homogeneous. The yields, specific activities, and purification factors obtained from this protocol are presented in Table I.

Table I: Purification of IPP Isomerase from JM101/pIPS241[a]

purification step	total act. (units) (% yield)	total protein (mg)	sp act. (units/mg)
crude extract	2970 (100)	511	5.8
DEAE-cellulose	2700 (90)	308	8.8
octyl-Sepharose	2400 (79)	120	20

[a] Purification based on approximately 7 g wet cell wt of JM101/pIPS241 (obtained from a 1-L culture in late log phase). Yields and activities are an average of four purifications.

Kinetic Measurements. IPP isomerase assays were performed according to the acid-lability procedure (Satterwhite, 1985). The assay mixture contained the following: 50 mM HEPES (pH 7.0), 200 mM potassium chloride, 10 mM magnesium chloride, 1 mg/mL BSA, 0.5 mM dithiothreitol, and 350 μM [1-^{14}C]IPP (2 μCi/μmol). The reaction was initiated by addition of enzyme, and incubation was continued for 10 min at 37 °C. The assays were quenched by addition of 0.2 mL of hydrochloric acid–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 ligroin (bp 90–120 °C), and a 0.5-mL sample of the ligroin layer was removed for counting. Rates of inactivation were determined by preincubating the enzyme with FIPP (0.5–5 μM) in assay buffer at 25 °C. At various time intervals, samples were removed from the inactivation mixture and diluted 12.5-fold into buffer containing 350 μM [1-^{14}C]IPP. The remaining isomerase activity was determined by the acid-lability assay. The percent inhibition was determined by comparing inhibited samples to noninhibited controls. The rate constants for inactivation (k_{inact}) were determined by fitting the percent remaining activity to a single-exponential time course with a nonlinear regression analysis (Enzfitter, Elsevier-Biosoft). The values of k_2 and K_I were determined by extrapolating the values of k_{inact} to saturating concentrations of inhibitor with the nonlinear form of the Michaelis–Menten equation (Enzfitter, Elsevier-Biosoft).

Stoichiometry of IPP Isomerase–Inhibitor Complexes. IPP isomerase (5–15 μg) was incubated at 25 °C with an excess of [4-^{3}H]FIPP (48 μCi/μmol) for 30 min to ensure complete labeling. The solutions were diluted to a final volume of 1 mL with 10 mM Tris-HCl (pH 7.4) and transferred to microconcentrators (Centricon 10, Amicon Corp.). The microconcentrators were spun at 5400*g* 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 IPP Isomerase. IPP isomerase (0.62 μmol) was inactivated with [4-^{3}H]FIPP as described above. Unbound inhibitor was removed by dialysis over a 24-h period against 3 × 2 L of 10 mM Tris-HCl, pH 8.0. The labeled protein (2.25 μCi/μmol) was precipitated by addition of 4 volumes of cold acetone and collected by centrifugation (7000*g*, 15 min). The protein pellet was dried under vacuum and dissolved in 8 M urea. The resulting

suspension was incubated at 50 °C to solubilize all of the protein. The denatured protein was digested with 1/20 (w/w) TPCK-treated trypsin (251 units/mg) for 20 h at room temperature in buffer containing the following: 50 mM Tris-HCl (pH 8.5), 0.1 mM calcium chloride, 1 mM magnesium chloride, 1 mM zinc chloride, 2 M urea, and 65 units of calf intestinal phosphatase.

Separation of Tryptic Peptides by Reversed-Phase High-Performance Liquid Chromatography (HPLC). The tryptic peptides from inactivated IPP isomerase were separated on a Vydac C-18 peptide column (0.46 × 25 cm) with a Waters 501 HPLC system equipped with a Waters Associates Model 441 absorbance detector. The eluate was monitored by UV absorbance at 214 nm and by detection of radioactivity with a Flo-One\β radioactive flow detector. Initial separation of the peptide mixture was accomplished under the following conditions [buffer A is 0.1% trifluoroacetic acid (TFA); buffer B is acetonitrile–water (3:2 v/v) containing 0.1% TFA]: The column was equilibrated with buffer A. Peptides were eluted with a linear gradient starting at 100% buffer A and decreasing to 30% buffer A over a period of 80 min. A flow rate of 1 mL/min was used, and fractions of 1 mL were collected. Three distinct regions of radioactivity were observed (I, II, and III). Fractions belonging to each region were combined and lyophilized. Peptides from region III were rechromatographed with a linear gradient commencing at 55% buffer A and decreasing to 45% buffer A over a period of 60 min. Under these conditions the peptides from region III were further separated into three closely migrating regions of radioactivity (III-A, III-B, and III-C). Fractions belonging to each region were combined and lyophilized. Final purification of the labeled peptides from region III was accomplished with a solvent system consisting of buffer A (10 mM ammonium acetate, pH 6.8) and buffer B [acetonitrile–buffer A (3:2 v/v)]. The combined fractions from each of the regions III-A, III-B, and III-C were rechromatographed separately with a shallow, linear gradient starting at 65% buffer A and decreasing to 50% buffer A over 60 min (flow rate = 1 mL/min). Fractions containing radioactivity were lyophilized and submitted for sequence analysis by automated Edman degradation on an Applied Biosystems Model 477A protein sequencer.

Determination of Free Cysteine. The number of free thiol groups per IPP isomerase monomer was determined with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959). IPP isomerase (sp act. 19 units/mg) was exchanged into buffer containing 100 mM Tris-HCl (pH 8.0) and 1 mM EDTA by diluting the enzyme with the buffer and reconcentrating in a microconcentrator (Centricon 10, Amicon Corp.). After each cycle of dilution and concentration, the filtrate was tested with DTNB, and the process was repeated until a color change was no longer observed. The final concentration of the protein was determined by its absorbance at 280 nm with an absorptivity index $\epsilon_{280}^{0.01\%} = 1.43$ previously determined for pure enzyme. Aliquots of the enzyme solution were added to a buffer containing 8 M urea, 100 mM Tris-HCl (pH 8.0), and 1 mM EDTA, to a final volume of 0.49 mL. A 10-μL aliquot of DTNB in dimethylformamide (10 mM) was then added and allowed to react at room temperature for 10 min. The absorbance of the solution was measured at 412 nm, and the concentration of the released thionitrobenzoate was determined with a molar extinction coefficient of 14 290 M^{-1} cm^{-1} (Russo & Bump, 1988).

Results

Expression of S. cerevisiae IPP Isomerase in E. coli. A prokaryotic expression vector for *S. cerevisiae* IPP isomerase was constructed by removing the transcription promoter/terminator regions from the native yeast gene and inserting the coding region of the gene into an *E. coli* expression vector. The strategy used in construction of the IPP isomerase reading-frame cassette is detailed in Figure 1. The plasmid pARC133B (Anderson et al., 1989) contains the native yeast gene for IPP isomerase as a 3.5-kb *Eco*RI insert in pBluescript SK(+). pARC133B has a unique *Mlu*I restriction site conveniently located three nucleotides downstream from the first stop codon of the reading frame. Cleavage at the *Mlu*I site and at a unique *Hin*dIII site situated downstream at the 3′-end of the *Eco*RI insert enabled the region of DNA containing the native yeast transcription terminator to be removed. Following treatment with Klenow fragment, a *Hin*dIII site was introduced with a synthetic linker, and ligation produced pARC133H, a 5.6-kb plasmid with the 3′-end of the reading frame adjacent to the multiple cloning site of pBluescript SK(+). Since no useful restriction sites were in the region immediately upstream of the translation initiation codon, PCR-mediated site-directed mutagenesis was used to create two new endonuclease recognition/cleavage sites (Kadowaki et al., 1989; Hemsley et al., 1989). The modifications positioned a unique *Nde*I restriction site (CATATG) at the translation initiation codon of the IPP isomerase gene and a new *Eco*RI site 16 nucleotides upstream from the translation initiation codon. A 1.75-kb *Xho*I fragment derived from pARC133H was used as the template for the PCR. This fragment contained 0.88 kb of yeast genomic DNA situated upstream from the IPP isomerase reading frame, 0.864 kb of the IPP isomerase reading frame, and 23 base pairs of the pBluescript multiple cloning site. The primers used in the PCR are described in Figure 1. The upstream primer was 32 nucleotides in length, and the structure was based on positions 431–462 (sense strand) of the published sequence for the IPP isomerase gene (Anderson et al., 1989). Mutations introduced by this primer were a single-base alteration at position 434 (A to G), which created the *Eco*RI restriction site, and a three-base mutation at positions 448–450 (TCA to CAT), which created the *Nde*I restriction site. The downstream primer was 28 nucleotides in length, corresponding to positions 661–688 (antisense strand) of pBluescript SK(+), and contained recognition/cleavage sites for *Xho*I, *Sal*I, and *Cla*I. The construction of the IPP isomerase reading-frame cassette was completed by digestion of the PCR fragments with *Xho*I and *Eco*RI, followed by ligation into the multiple cloning site of pBluescript SK(+).

Previous reports (Newton et al., 1988; Tindall & Kunkel et al., 1988; Dunning et al., 1988) indicated that the frequency of replication errors made by *Taq* polymerase can be significant. To ensure that no unwanted mutations had been introduced into the IPP isomerase reading frame, the insert was sequenced by the dideoxynucleotide chain termination procedure (Sanger et al., 1977). Except for mutations deliberately introduced on the upstream PCR primer, the IPP isomerase reading frame was unaltered from the DNA sequence previously described by Anderson et al. (1989).

The vector used for overproduction of IPP isomerase (pARC306N) is designed for the direct cloning and expression of eukaryotic genes in *E. coli*. This plasmid contains the highly expressed *rec 7* promoter, which is a hybrid construct based on the *E. coli rec A* promoter and the phage T7 gene *10* leader sequence (Olins et al., 1988). The gene *10* leader sequence contains a ribosome binding site and a translation intiation codon (ATG) which forms part of the recognition/cleavage site for *Nde*I (CATATG). To stabilize vector–host relation-

Table II: Expression of IPP Isomerase in *E. coli*

strain	growth temp (°C)	induction[a]	sp act. (units/mg)	relative sp act.
JM101	37	-	2.8×10^{-5}	1
JM101/pIPS241	37	-	3.1	1.1×10^5
JM101/pIPS241	37	+	2.1	7.5×10^4
JM101/pIPS241	30	+	1.8	6.4×10^4
JM101/pIPS241	30	-	0.5	1.7×10^4

[a] Cultures were grown to early log phase before induction with nalidixic acid (50 μg/mL); incubation was continued for a further 2–4 h before the cells were harvested. Noninduced cultures were grown to late log phase before harvesting.

ships (Gentz et al., 1981) and terminate transcription from the *rec 7* promoter, a strong transcription terminator was also included in pARC306N. The terminator is positioned downstream from a multiple cloning site, which also contains the translation start signal and the unique *NdeI* restriction site.

The IPP isomerase reading frame was cloned into pARC306N as a 0.87-kb *NdeI*/*SalI* fragment, and the resulting plasmid (pIPS241, 3.39 kb) was used to transform *E. coli* strain JM101. Cultures of the transformants and the untransformed host strain were grown to late log phase and then assayed for IPP isomerase activity. The results are summarized in Table II. In cultures carrying pIPS241, the specific activity of IPP isomerase was over 100000-fold greater than in the untransformed host strain, thus demonstrating that the eukaryotic IPP isomerase gene can be expressed at high levels in *E. coli*.

Constructs containing the *E. coli rec A* promoter are normally repressed in *rec A*$^+$ host strains (such as JM101) but can be induced by addition of nalidixic acid (Feinstein et al., 1983). This is also true for plasmids containing the *rec 7* promoter (Olins et al., 1988). However, little or no repression of the *rec 7* promoter was observed for transformants of JM101 carrying pIPS241, under a variety of different growth conditions. The results are summarized in Table II. Cultures were grown to early log phase ($OD_{590} \approx 1$) at a temperature of 30 or 37 °C. Nalidixic acid was then added, and the incubation was continued for an additional 2–4 h before the cells were harvested and assayed for IPP isomerase activity. The levels of enzyme activity in the induced cultures were compared with those of cultures that had been grown at the same temperature to late log phase, but without the addition of nalidixic acid. For cultures grown at 37 °C, the levels of IPP isomerase activity were found to be approximately the same for both induced and noninduced cells. For cultures grown at 30 °C, addition of nalidixic acid resulted in a small increase (3.6-fold) in the levels of IPP isomerase activity.

Examination of cell-free extracts prepared from cultures carrying pIPS241 by SDS–PAGE showed a protein that migrated with an apparent molecular weight of 39000 and that comigrated with an authentic sample of IPP isomerase partially purified from yeast (Figure 2A). This protein was not present in cell-free extracts prepared from the untransformed host strain. The molecular weight of 39000 determined here is in good agreement with those determined previously by SDS–PAGE for the native protein purified from yeast (Anderson et al., 1989; Reardon & Abeles, 1986).

Purification and Characterization of Recombinant IPP Isomerase. Cultures of JM101/pIPS241 used for overproduction of IPP isomerase were grown at 37 °C, without addition of nalidixic acid, and cells were harvested during late log phase/early stationary phase (OD_{590} = 3.7–4.5). IPP isomerase was purified from the crude cell-free extract in two steps: ion exchange chromatography on DEAE-cellulose,

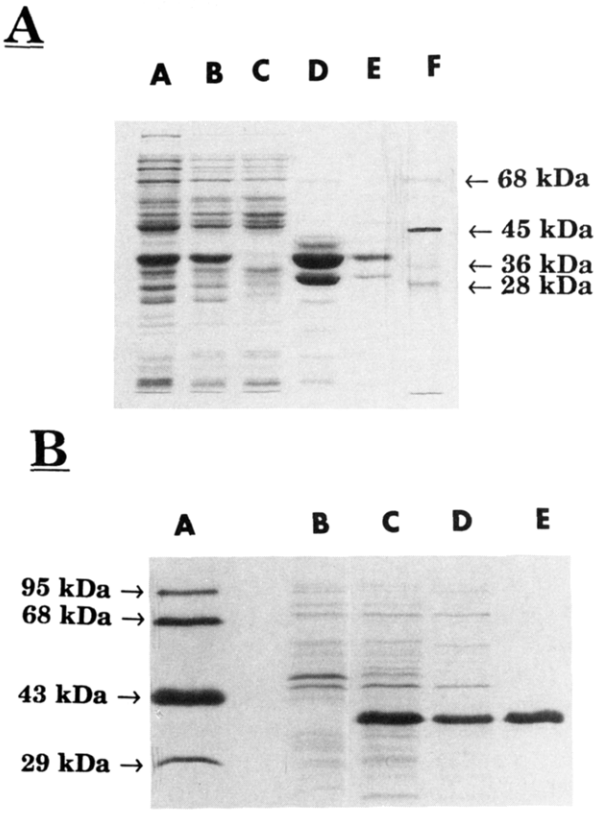


FIGURE 2: Comparison of crude cell-free extracts prepared from IPP isomerase overproducing strains of JM101. (A) A 12% acrylamide–SDS gel of the following crude cell-free extracts: (lane A) JM101/pIPS241 induced with 50 μg/mL nalidixic acid (10 μg); (lane B) JM101/pIPS241, noninduced culture (10 μg); (lane C) JM101 (10 μg); (lane D) partially purified IPP isomerase from yeast (10 μg); (lane E) mixture of the samples from lanes B and D (2 μg each); (lane F) molecular weight standards. (B) Purification of recombinant IPP isomerase: (lane A) molecular weight standards; (lane B) JM101 (10 μg); (lane C) crude cell-free extract prepared from JM101/pIPS241 (7 μg); (lane D) after chromatography on DE52 (5 μg); (lane E) after chromatography on octyl-Sepharose (3 μg).

followed by hydrophobic interaction chromatography on octyl-Sepharose. This procedure yielded protein that gave a single band by SDS–PAGE and was judged to greater than 95% pure (Figure 2B). The specific activity of the pure enzyme was 20 units/mg. This value is 7.5-fold greater than the specific activity of IPP isomerase isolated from yeast with the procedure of Reardon and Abeles (1986). The value of K_m determined for IPP was 43 μM, in good agreement with the previously determined value of 35 μM (Reardon & Abeles, 1986).

Inactivation of IPP Isomerase by FIPP. FIPP is a selective active-site-directed inhibitor of IPP isomerase from *C. purpurea* (Muehlbacher & Poulter, 1988). Inactivation occurs by an S_N2 displacement of fluoride by an enzymatic nucleophile to produce an inactive, covalent enzyme–inhibitor complex (Muehlbacher et al., 1989).

The kinetic parameters for inactivation of the recombinant yeast IPP isomerase by FIPP were determined by incubating the enzyme with varying concentrations of the inhibitor. This treatment caused a time-dependent, pseudo-first-order loss of enzyme activity. The rate constants for inactivation (k_{inact}) were determined from a semilogarithmic plot of residual activity versus time. The rate constant for inactivation at saturating levels of inhibitor (k_2) and the inhibition constant (K_I) were determined from a replot of k_{inact}^{-1} versus $[FIPP]^{-1}$. The values of k_2 and K_I were 1.0 ± 0.02 min^{-1} and 3.8 ± 1.6 μM, respectively. As expected for an active-site-directed process,

Table III: Tryptic Peptides Labeled by [4-^{3}H]FIPP

peptide	length (residues)	sequence[a]	dpm (%)[b]
III-A1	31	ATEKITFPDLWTNTCCSHPLCIDDELGLKGK	7.1
III-A2	27	ITFPDLWTNTCCSHPLCIDDELGLKGK	8.7
III-B1	29	ATEKITFPDLWTNTCCSHPLCIDDELGLK	32.2
III-B2	25	ITFPDLWTNTCCSHPLCIDDELGLK	39.2
III-C1	29	ATEKITFPDLWTNTCCSHPLCIDDELGLK	3.0
III-C3	25	ITFPDLWTNTCCSHPLCIDDELGLK	4.0

[a] The residue underlined indicates the position of the radiolabeled amino acid. [b] Percentage of the total radioactivity associated with the peptide.

Scheme II: Potential Trypsin Cleavage Sites between Residues 122 and 158

---QR ↓ ATEK ↓ ITFPDLWTNTCCSHPLCIDDELGLK ↓ GK ↓ LD---

the rate of inhibition of FIPP decreased in the presence of substrate. At a concentration of 5 μM FIPP, the half-life of inhibition was 1.2 min. At the same concentration of inhibitor, but in the presence of 350 μM IPP, the half-life of inhibition increased to 11 min.

The stoichiometry of the FIPP–IPP isomerase complex was determined with radiolabeled FIPP. Samples of IPP isomerase were inactivated with an excess of radiolabeled inhibitor, and unbound inhibitor was removed by repeated washing on an ultrafiltration membrane. We found that 0.79 ± 0.08 mol of FIPP was bound per mole of IPP isomerase.

To determine the site of covalent attachment in the enzyme–inhibitor complex, samples of IPP isomerase were inactivated with radiolabeled FIPP, and the labeled enzyme was denatured and digested with trypsin. To ensure efficient chromatography of the labeled peptides, it was found necessary to remove the diphosphate moiety from the inhibitor by treatment of the digestion mixture with alkaline phosphatase. Initial separation of the resulting tryptic peptides by HPLC at low pH (Figure 3A) gave three areas of radioactivity, designated I, II, and III. Fractions I and II eluted with, or just after, the void volume of the column and contained approximately 24% of the total radioactivity in the sample. Fast atom bombardment mass spectral analysis of the components from peaks I and II failed to identify any labeled peptide fragments, and characterization of these fractions was not pursued further. The remaining 76% of the radioactivity eluted in a broad region between 40 and 45% buffer A (region III). This region contained several UV-active peaks and was further purified by rechromatography in the same low-pH buffer with a shallower gradient (Figure 3B). Under these conditions region III resolved into three closely migrating components, designated III-A, III-B, and III-C. Of the total radioactivity contained in region III, 10% was isolated in region III-A, 72% in region III-B, and 17% in region III-C. Final purification of the radiolabeled peptides was achieved by rechromatography of each of these regions at pH 6.8 (Figure 4). A total of eight separate radioactive fractions were obtained. Insufficient quantities of peptides represented by III-C2 and III-C4 were obtained for sequencing. The remaining six fragments were sequenced, and their structures are given in Table III. Each was a derivative of the 25-residue peptide I-T-F-P-D-L-W-T-N-T-*-*-S-H-P-L-*-I-D-D-E-L-G-K. This sequence exactly matches positions 128–152 of the presumptive amino acid sequence of IPP isomerase. The residues in cycles 11, 12, and 16 were assigned as cysteine residues by inference from the known amino acid sequence. The potential trypsin cleavage sites located between residues 124 and 160 in the amino acid sequence of IPP isomerase are shown in Scheme II. Peptides III-B2 and III-C3 each contained the basic 25 amino acid sequence, while III-B1 and III-C1 had a four amino acid

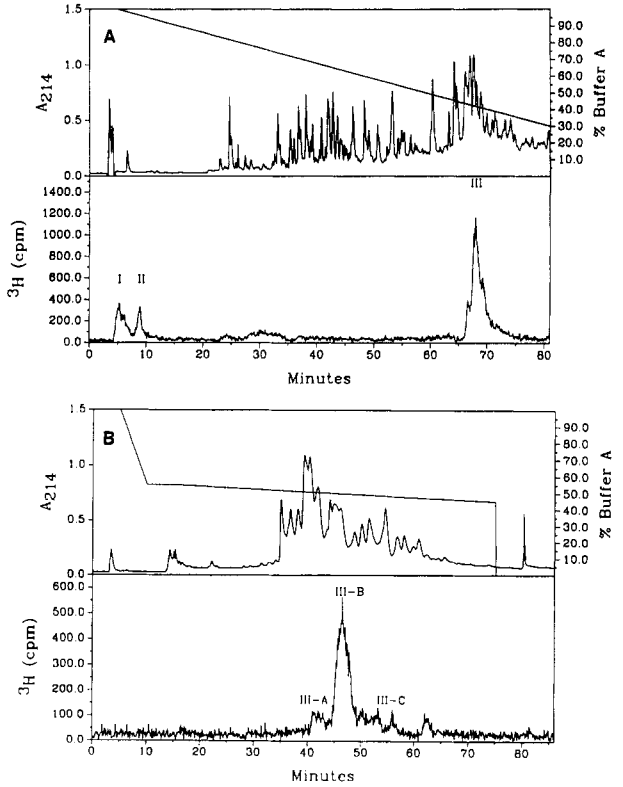


FIGURE 3: Initial separation of tryptic peptides from [4-^{3}H]FIPP-labeled IPP isomerase. [4-^{3}H]FIPP-labeled IPP isomerase was denatured and digested with TCPK-treated tryspin as described under Materials and Methods. (A) Initial separation of the tryptic peptides on a Vydac C-18 HPLC column, using the following solvent system: buffer A, 0.1% TFA in water; buffer B, acetonitrile–buffer A, 3:2. (B) Rechromatography of the peptides eluting in region III.

extension (A-T-E-K) at the amino terminus of the 25-mer. Peptide III-A2 had a two amino acid (G-K) extension attached at the carboxy terminus, while peptide III-A1 possessed both the amino- and carboxy-terminal extensions. Peptides III-B1 and III-C1 possessed the same amino acid sequence but different chromatographic mobilities. This might be due to incomplete hydrolysis of the diphosphate group in the covalently attached inhibitor by alkaline phosphatase.

With the exception of peptide III-C3, a burst of radioactivity was observed upon elution of the second cysteine residue during the sequencing of each peptide. This residue corresponds to Cys-139 in the amino acid sequence of IPP isomerase. In peptide III-C3, radioactivity was associated with Cys-138, but this only represents a 4.0% of the total radioactivity recovered from inactivated enzyme.

Quantitation of the Free Thiol Groups in IPP Isomerase. With the identification of Cys-139 as the primary site of covalent attachment in the enzyme–inhibitor complex, it became important to determine whether this residue might normally be involved in the formation of a disulfide bridge. IPP isomerase contains seven cysteine residues per enzyme monomer (Anderson et al., 1989), and a quantitative deter-

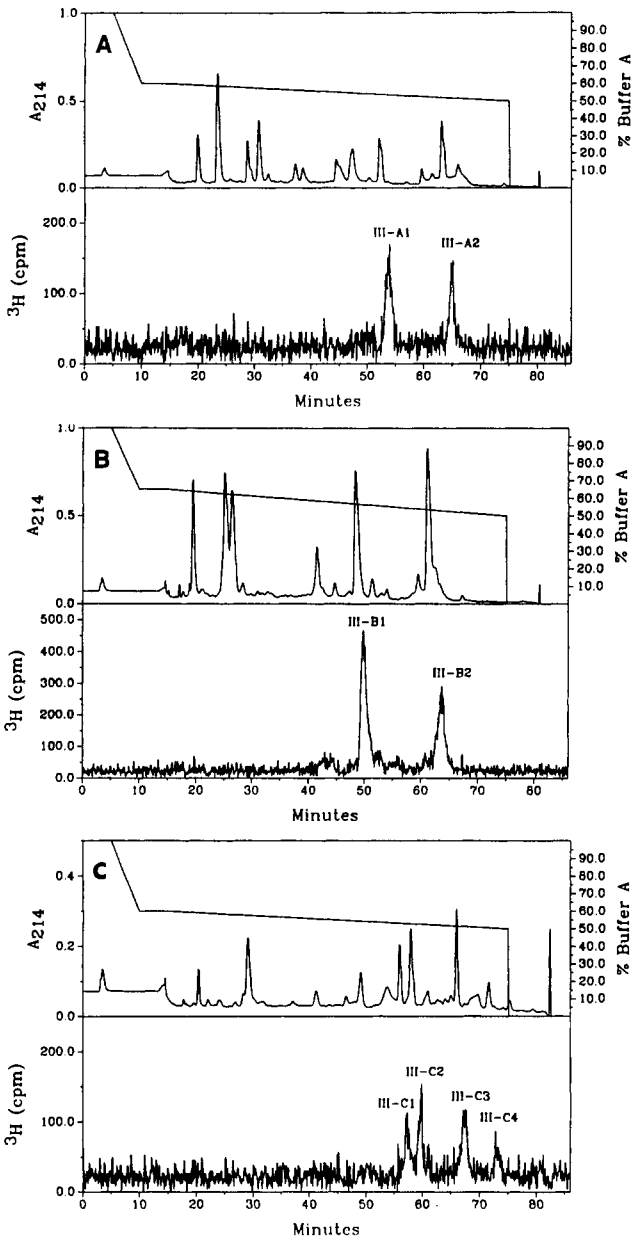


FIGURE 4: Final purification of the [4-^{3}H]FIPP-labeled tryptic peptides. The following solvent systems was used: buffer A, 10 mM ammonium acetate (pH 6.8); buffer B, acetonitrile–buffer A, 3:2. (A) Rechromatography of the peptides from region III-A. (B) Rechromatography of the peptides from region III-B. (C) Rechromatography of the peptides from region III-C.

mination of the number of free thiols was made with the colorimetric reagent DTNB (Ellman, 1959). IPP isomerase (sp act. 19 units/mg) was transferred to a buffer containing 0.1 M Tris-HCl (pH 8.0) and 1 mM EDTA and denatured in 8 M urea prior to addition of an equivalent excess of DTNB. It was found that 6.8 ± 0.4 mol of the thionitrobenzoate anion was released per mole of enzyme monomer, thus demonstrating that all of the cysteine groups in active IPP isomerase are free to react with DTNB and effectively excluding the possibility that Cys-139 might be part of a disulfide bridge.

Discussion

We were able to obtain high levels of expression of yeast IPP isomerase in *E. coli* by cloning the reading frame of the gene from *S. cerevisiae* into a prokaryotic expression vector containing a synthetic *rec 7* promoter. Specific activities of 6–7 units/mg for IPP isomerase were rountinely obtained in crude cell-free extracts prepared from the overproducing strains. This corresponds to IPP isomerase levels of approximately 30–35% of total soluble protein. The recombinant enzyme can be purified to greater than 95% homogeneity by a simple two-step procedure, which can easily be completed in a single day. The specific activity of the purified enzyme was 20 units/mg, a 7.5-fold enhancement over the specific activity of IPP isomerase purified from yeast (Reardon & Abeles, 1986). Undoubtedly, the higher specific activity is due to the simpler purification procedure for the overproduced enzyme. IPP isomerase is relatively unstable and looses activity if subjected to many of the manipulations used for the purification from yeast. With respect to its kinetic parameters and migration on SDS gels, the recombinant enzyme appears to be identical with IPP isomerase from yeast.

There is a significant discrepancy between the molecular mass of IPP isomerase of 33 350 daltons deduced from the gene sequence and values of 39 000–40 000 daltons determined by SDS–PAGE by us (Anderson et al., 1989) and by Reardon and Abeles (1986). Anderson et al. proposed the difference might be due to a posttranslational modification, perhaps glycosylation. Indeed, IPP isomerase contains potential glycosylation sites at Asn-5, Asn-22, and Asn-221. However, in experiments designed to detect glycosyl residues commonly found in *S. cerevisiae*, they were unable to find any evidence to suggest that IPP isomerase was glycosylated. They concluded that the discrepancy in molecular mass was most likely due to a gel artifact. This conclusion is corroborated by the data presented here. The recombinant enzyme comigrates with an authentic sample of partially purified IPP isomerase from yeast, and it is very unlikely that similar posttranslational modifications occurred in *S. cerevisiae* and *E. coli*.

FIPP is a specific active-site-directed inhibitor of IPP isomerase from *C. purpurea* (Muehlbacher & Poulter, 1988). The data presented here demonstrated that this compound is also an effective active-site-directed inhibitor of IPP isomerase from *S. cerevisiae*. Inactivation occurs by formation of a covalent enzyme–inhibitor complex with a stoichiometry of approximately 1:1. The primary site of covalent attachment is Cys-139. Substrate protection experiments indicate Cys-139 is located in the active site of the enzyme, probably within the isoprene binding domain. It was previously reported that IPP isomerase is inactivated by thiol-selective reagents (Agarnoff et al., 1960) and proposed that the thiol group is located in the active site (Shah et al., 1965; Reardon & Abeles, 1986).

Reardon and Abeles (1986) also presented evidence that a thiol group may be involved in catalysis. They found that 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. The pK value determined for this thiol group was similar to the pK value of 9.4 they measured for the alkaline limb of a pH/rate profile for the enzyme. IPP isomerase from *C. purpurea* is also inactivated by iodoacetamide (Muehlbacher & Poulter, 1988). However, in contrast to the results obtained with the yeast enzyme, saturating concentrations of substrate did not protect against inactivation, and enzyme inactivated by iodoacetamide also reacted with FIPP. These observations indicate that blocking the reactive thiol group in the *Claviceps* enzyme did not preclude the binding of FIPP in the active site.

The identification of Cys-139 as the nucleophile responsible for reaction with FIPP, protection by IPP, and demonstration that Cys-139 is not part of a disulfide bridge in active enzyme provide strong evidence that the thiol group is located within the active site of yeast IPP isomerase. Recent mechanistic

studies (Muehlbacher & Poulter, 1988; Reardon & Abeles, 1986) suggest a carbocation mechanism which requires protonation of the unactivated carbon–carbon double bond as the first step in isomerization. The anatarafacial [1.3] isomerization requires functional groups capable of catalyzing a protonation–deprotonation on both faces of the hydrocarbon moiety in the substrate. The role that a thiol group might play in this mechanism is not clear and is the subject of further study in this laboratory.

ACKNOWLEDGMENTS

We are grateful to Jerriann Ernstsen, Hazel Coffman, and Bobby Huang for their technical assistance with the protein purification and to Bob Shackman for sequencing the radiolabeled peptides. We thank Dr. Mike Bittner for supplying pARC306N and helpful advice.

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CORRECTION

Slow Binding Inhibition of 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase, by Pearl Louis-Flamberg, Catherine E. Peishoff, Deborah L. Bryan, Jack Leber, John D. Elliott, Brian W. Metcalf, and Ruth J. Mayer*, Volume 29, Number 17, May 1, 1990, pages 4115–4120.

Page 4118. In Table II, part B, k_{on} for compound **1** should read 16 (0.3).