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Isopentenyl Diphosphate:Dimethylallyl Diphosphate Isomerase

AN IMPROVED PURIFICATION OF THE ENZYME AND ISOLATION OF THE GENE FROM *SACCHAROMYCES CEREVISIAE**

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Isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IPP isomerase) is an enzyme in the isoprenoid biosynthetic pathway which catalyzes the interconversion of the primary five-carbon homoallylic and allylic diphosphate building blocks. We report a substantially improved procedure for purification of this enzyme from *Saccharomyces cerevisiae*. An amino-terminal sequence (35 amino acids) was obtained from a highly purified preparation of IPP isomerase. Oligonucleotide probes based on the protein sequence were used to isolate the structural gene encoding IPP isomerase from a yeast λ library. The cloned gene encodes a 33,350-dalton polypeptide of 288 amino acids. A 3.5-kilobase *Eco*RI fragment containing the gene was subcloned into the yeast shuttle vector YRp17. Upon transformation with plasmids containing the insert, a 5–6-fold increase in IPP isomerase activity was seen in transformed cells relative to YRp17 controls, confirming the identity of the cloned gene. This is the first reported isolation of the gene for IPP isomerase.

The isoprenoid biosynthetic pathway is ubiquitous and produces a wide variety of small, usually lipophilic molecules which fulfill a number of important functions (Spurgeon and Porter, 1981). Prominent examples include sterols (structural components of eukaryotic membranes, hormones), carotenes (photoreceptors in visual and photosynthetic systems), glyceryl ethers (archaebacterial membranes), respiratory coenzymes, insect juvenile hormones, cytokinins (plant hormones), and phosphorylated polyprenols (sugar carriers in biosynthesis of bacterial cell wall polysaccharides and eukaryotic glycoproteins). Isoprenoids are constructed in two distinct building phases. The first is the stepwise assembly of (*S*)-3-hydroxymethylglutaryl-coenzyme A (CoA)[1] from three molecules of acetyl-CoA by a Claisen and an aldol condensation. The second encompasses a complex set of alkylation reactions between nucleophilic acceptors and electrophilic allylic isoprenoid diphosphates catalyzed by prenyltransferases, a family of enzymes responsible for construction of polyisoprenoid chains and attaching these chains to a variety of isoprenoid and non-isoprenoid acceptors (Poulter and Rilling, 1981a). Fig. 1 illustrates the early parts of the pathway common to all organisms, and the ultimate metabolites commonly found in mammals.

SCHEME 1

Dimethylallyl diphosphate (DMAPP) is the primer for all subsequent prenyl transfer reactions. The potent electrophile is created from isopentenyl diphosphate (IPP) through a [1.3] transposition of hydrogen catalyzed by IPP:DMAPP isomerase (EC 5.3.3.2) (Poulter and Rilling, 1981b) as shown in Scheme 1. Although i-somerase activity is ubiquitous, little is known about how the enzyme is regulated, no isomerase mutants are known for any organism, and significant purifications of the enzyme have only been reported from three sources, porcine liver (Banthorpe *et al.*, 1977; Bruenger *et al.*, 1986), *Claviceps* (Bruenger *et al.*, 1986; Muehlbacher and Poulter, 1988), and *Saccharomyces cerevisiae* (Reardon and Abeles, 1986). The structure of the enzyme has not been characterized. Mechanistic studies indicate that isomerization occurs by a protonation-deprotonation sequence involving a highly electrophilic carbocationic species (Muehlbacher and Poulter, 1988; Reardon and Abeles, 1986). Similar enzyme-catalyzed protonations of unactivated carbon-carbon double bonds rarely occur in nature.

We now report an improved procedure for purification of IPP isomerase which was developed as part of a study of the mechanism of action of the enzyme (Muehlbacher and Poulter, 1988; Muehlbacher *et al.*, 1989). The amino-terminal sequence of the enzyme was determined and used to design oligonucleotide probes for the isolation of clones containing the IPP isomerase gene from a yeast genomic DNA library. The identified gene encodes a 33,350-dalton protein whose NH_2-terminal sequence matched that of IPP isomerase. Yeast transformants containing multiple extrachromosomal copies of the IPP isomerase gene gave a 5-fold increase in IPP isomerase activity above controls.

EXPERIMENTAL PROCEDURES

Materials

[1-^{14}C]Isopentenyl diphosphate (IPP) was purchased from Amersham Corp. Ultrapure ammonium sulfate was obtained from

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05090.

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[1] The abbreviations used are: CoA, coenzyme A; BME, 2-mercaptoethanol; DMAPP, dimethylallyl diphosphate; endo H, β-*N*-acetylglucosaminidase H; HPLC, high pressure liquid chromatography; IPP, isopentenyl diphosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kb, kilobase.

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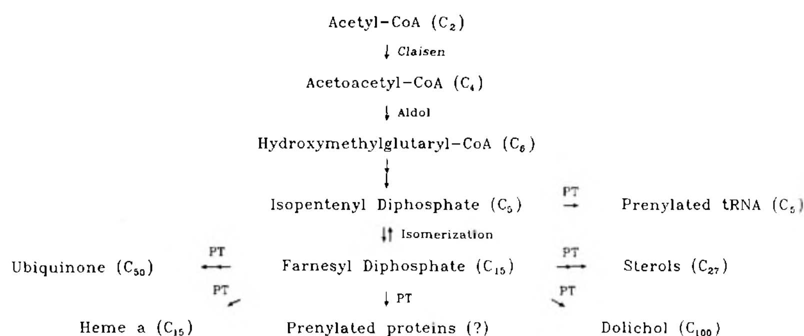

FIG. 1. **An outline of the building reactions in the isoprenoid pathway.** The steps through isomerization of IPP to DMAPP are common to all organisms. The prenyltransfer steps (*PT*) requiring electrophilic allylic diphosphates shown are those commonly encountered in mammals. The number of carbons refers to those derived from the isoprene pathway in each metabolite or class of metabolites. Processing reactions in sterol metabolism commonly remove three isoprenoid carbons from lanosterol (C_{30}).

Schwartz/Mann. Microcrystalline DE52 ion exchange resin was from Whatman, and PBE 94 Polybuffer Exchanger and Polybuffer 74 were from Pharmacia LKB Biotechnology Inc. *n*-Butylsepharose was prepared as reported by Woodside (1987). Fleischmann's yeast paste was purchased from Western Standard Distributing Company, Salt Lake City, UT. Radioactivity was measured in Optifluor scintillation media (Packard Instrument Company) using a Packard Tricarb model 4530 liquid scintillation spectrometer.

Strains, Media, and Growth Conditions

The yeast strain used was JGY195 (*MATα*, *ade*5, *gal*7Δ102, *leu*2–3, *leu*2–112, *trp*1–289, *ura*3–52; J. Yarger, Amoco Technology Co.), *Escherichia coli* strains DH5α (F^-, φ80d*lac*ZΔM15, *end*A1, *rec*A1, *hsd*R17 (r_K^-,m_K^-, *sup*E44, *thi*-1, λ^-, *gyr*A96, *rel*A1; Bethesda Research Laboratories) and JM101 (Δ(*lac-pro*AB), *supE*, *thi*/F′, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15; Yanisch-Perron *et al.*, 1985) were used for routine cloning procedures. λ-phage were propagated in *E. coli* strain LE392 (F^-, *hsd*R514 (r_K^-,m_K^-), *sup*E44, *sup*F58, *lac*Y1 or Δ(*lac*IZY)6, *gal*K2, *gal*T22, *met*B1, *trp*R55, λ^-; Maniatis *et al.*, 1982). Untransformed yeast strains were grown at 30 °C in YEPD media (Sherman *et al.*, 1986). Yeast strains harboring plasmids were grown in SD synthetic complete minimal media (Sherman *et al.*, 1986) lacking uracil (*ura3* selection).

General Procedures

Plasmid preparations, transformations, and other standard molecular biology techniques were carried out as described (Maniatis *et al.*, 1982). Transformations of yeast were performed by the lithium acetate procedure (Sherman *et al.*, 1986).

Ion exchange chromatography, gel exclusion chromatography, and affinity chromatography of proteins were conducted at 4 °C. HPLC was carried out at 25 °C on a Waters system. Dialysis was performed in Spectrapor dialysis bags (25.5 mm, M_r cutoff 6000–8000), or in a Micro-ProDicon model 115 forced dialysis concentrator using Pro-Dimem PA-10 dialysis membranes (M_r cutoff 10,000). Concentration of protein solutions was performed in an Amicon stirred ultrafiltration cell (50 ml capacity) using a PM-10 filter (M_r cutoff 10,000). SDS-polyacrylamide gel electrophoresis of proteins was performed using the discontinuous buffer system of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R or silver nitrate (Merril *et al.*, 1981). Protein was measured by the method of Bradford (Scopes, 1982) using bovine serum albumin as standard. Isomerase assays were performed using the acid lability procedure (Satterwhite, 1985).

Purification of IPP Isomerase

All enzyme isolation procedures were at 4 °C except HPLC chromatographies, which were at 25 °C. Plasticware was used throughout the purification. We performed the following steps using cells disrupted either by sonication or with a bead mill. Both procedures involved significant heating despite constant immersion in ice and frequent breaks to recool the solutions. Yields from sonicated material were half that of the bead mill in initial extracts. The enzyme analyzed for NH_2-terminal sequence was isolated from cells disrupted by the sonication procedure.

Bead Mill Procedure—To a 250-ml bead-beater vessel (Biospec Products, Bartlesville, OK) was added 4 oz of 0.5-mm beads. The container was topped with a yeast suspension containing 1.5 kg of baker's yeast paste suspended in 1.5 liters of 100 mM potassium phosphate, pH 7.0, 10 mM BME, 1 mM phenylmethylsulfonyl fluoride, and cycled for 8 min, 15 s on and 15 s off, using an ice water jacket for cooling. The disrupted material was held on ice while the procedure was repeated until all of the suspension had been processed. The pH of the combined extracts was adjusted to 5.0 with 3.5 M acetic acid and stirred for 10 min. The precipitated material was removed by centrifugation at 11,000 × *g* for 45 min. The recovered supernatant was adjusted to pH 7.0 with 1.5 M ammonium hydroxide and fractionated with ammonium sulfate as described below.

Sonication Procedure—Fleischmann's yeast paste (1.5 kg) was suspended in 1.5 liter of extraction buffer as described above and divided into 4 × 750 ml batches. The batches were sonicated (Bronson Sonifier[R] Cell Disruptor model 350 equipped with a high gain disruptor ¾-inch horn), while being cooled in ice water, at a power setting of 7 and a duty cycle setting of 70% for 40 min. The pH was adjusted to 5.0 with 3.5 M acetic acid, and the suspension was stirred for 10 min. The precipitated material was removed by centrifugation at 11,000 × *g* for 45 min. The pH of the supernatant was adjusted to 7.0 with 1.5 M ammonium hydroxide.

The total volume of clarified extracts produced by either bead milling or sonication was 1700 ml. The pellet obtained by precipitation with ammonium sulfate (45–75% fraction) was dissolved in a minimal amount of 100 mM potassium phosphate, 10 mM BME, pH 7.0, buffer and dialyzed overnight against 4 × 4 liter changes of the same buffer.

The solution was loaded onto a 5.0 × 30-cm column of DE52 cellulose equilibrated with 100 mM potassium phosphate, 10 mM BME, pH 7.0. The column was washed with starting buffer (approximately 900 ml) until the UV absorbance of the eluate at 280 nm returned to the base line. IPP isomerase was eluted with a linear gradient, 3000 ml of 100–500 mM potassium phosphate, 10 mM BME, pH 7.0. Fractions of 25 ml were collected. Those containing activity (79–89) were pooled, saturated with ammonium sulfate, and centrifuged. The pellet was dissolved in a minimal amount of 5 mM potassium phosphate, 10 mM BME, pH 7.0, and dialyzed overnight against 4 × 2 liter changes of the same buffer.

A 5 × 21-cm *n*-butyl-Sepharose column was equilibrated with 5 mM potassium phosphate, 10 mM BME, pH 7.0. Protein from two DE52 chromatographies was loaded in starting buffer and eluted with a linear gradient (2000 ml) of 5–100 mM potassium phosphate, 10 mM BME, pH 7.0. Fractions of 17 ml were collected. Those with the highest activity (34–50) were pooled and concentrated to a final volume of 6 ml in an Amicon-stirred ultrafiltration cell fitted with a PM-10 membrane. The concentrated solution was dialyzed against 2 × 2 liter changes of 10 mM potassium phosphate, 2 mM dithiothreitol, pH 7.0.

A 1.5 × 27-cm column of PBE 94 exchanger (Pharmacia LKB Biotechnology Inc.) was equilibrated with 25 mM piperazine-HCl, 10 mM BME, pH 5.5. Enzyme was loaded through an injection loop and eluted with 1000 ml of 1:10 diluted Polybuffer 74 (Pharmacia LKB Biotechnology Inc.), 10 mM BME, pH 7.0. Fractions of 5 ml were collected. Those with the highest activity (20–30) were pooled, saturated with ammonium sulfate, stirred for 60 min, and centrifuged. The pellet was dissolved in a minimal amount of 10 mM potassium phosphate, 2 mM dithiothreitol, pH 7.0, to a final volume of 3 ml.

A 1.5 × 80-cm column of Sephacryl S-200 was equilibrated with 25 mM potassium phosphate, 100 mM NaCl, 10 mM BME, pH 7.0. The protein was loaded through an injector loop and eluted with the same buffer. Fractions of 2 ml were collected, and those with the highest activity (19–24) were combined, concentrated, and dialyzed in a Micro-ProDicon concentrator against 1 liter of 25 mM potassium phosphate, 5 mM dithiothreitol, pH 7.0, to give a final volume of 1.0 ml.

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A 0.75 × 7.5-cm Protein Pak DEAE-5PW column was equilibrated with 10 mM potassium phosphate, pH 7.0. The protein was loaded onto the column through a 5 ml loop injector, and separation of IPP isomerase from most remaining contaminants (including a reddish brown material) was achieved with the following potassium phosphate gradient run at pH 7.0: 0–5 min, 10 mM, pH 7.0; 5–20 min, 10–300 mM (linear); 20–50 min, 300 mM (isocratic). Fractions of 0.35 ml were collected, and those with the highest activity (10–15) were pooled, concentrated, and dialyzed in a Micro-ProDicon concentrator against 1 liter of 25 mM potassium phosphate, 5 mM dithiothreitol, pH 7.0, to give a final volume of 0.7 ml. Samples of this preparation were stored at −70 °C for periods of longer than 3 weeks without significant loss of activity. SDS-gel electrophoresis of this preparation revealed a major protein band at approximately 39,000 daltons judged to be greater than 95% pure.

Cloning of the IPP Isomerase Gene

A sample of the purified protein was submitted to the University of Illinois Biotechnology Center for NH_2-terminal Edman degradation. This analysis gave a 35-amino acid sequence in which cycle 9 was indeterminate, cycle 22 was either Ile or Gln, and cycles 32–35 were less certain. This sequence was used to construct the two single-stranded DNA probes shown in Fig. 2. The oligonucleotide probes were 5′ end-labeled with [γ-^{32}P]ATP and used to screen a yeast genomic library constructed in the λ vector MG14 by cloning partial *Sau*3AI yeast DNA fragments into *Bam*HI vector arms lacking *Eco*RI and *Hin*dIII sites (Olson *et al.*, 1986).

DNA from 10,000 plaques of this library was transferred to each of four sets of nitrocellulose filters, and each probe described above was hybridized to two sets of filters under low stringency conditions (0.9 M NaCl, 90 mM sodium citrate, pH 7.4, 5 × Denhardt's solution, 0.1% SDS, 100 μg/ml salmon sperm DNA, and 100 μg/ml wheat germ tRNA at 40 °C for 10 h). Ten plaques were found which hybridized to both probes. After plaque purification and rescreening, Southern analysis of the phage DNAs revealed that most contained a 3.5-kb *Eco*RI fragment that hybridized to both probes. Other phage DNAs produced a hybridizing *Eco*RI fragment corresponding to one arm of the λ vector. The 3.5-kb *Eco*RI fragment was ligated into the vector pBluescript SK(+) (Stratagene) in both orientations. The resulting phagemids were designated pARC133A and pARC133B.

Sequencing Methods

DNA sequence analysis of the IPP isomerase gene was performed by the dideoxy-chain termination method of Sanger *et al.* (1977) using the Sequenase® kit (U.S. Biochemicals). Initial sequencing reactions were carried out using a subset of nucleotides present in probe 1 as a sequencing primer. Computer-assisted analysis of sequence data was performed using the PC Gene programming package (Intelligenetics Inc., Mountainview, CA). Strain JM101 was used as the host for phagemid (pBluescript SK(+))-based plasmids. Helper phage R408 (Stratagene) was used as directed by the supplier to produce single-stranded phagemid DNA.

Expression of the IPP Isomerase Gene

A 3.5-kb *Eco*RI fragment from pARC133A was subcloned into the yeast shuttle vector YRp17. The resulting plasmids with the insert in both directions, pMA150A and pMA150B, were used to transform yeast strain JGY195 to uracil prototrophy. The transformants were grown as described above. Samples were harvested by centrifugation and assayed for IPP isomerase activity.

Incubation of IPP Isomerase with Endo H

Incubations of native isomerase with endo H were conducted essentially as described by Julius *et al.* (1984). Reactions contained 50 μg of IPP isomerase, 10 milliunits of endo H in 0.3 M sodium citrate buffer, pH 5.5, and were incubated at 37 °C for 20 h prior to analysis by SDS-PAGE. Samples of denatured IPP isomerase were prepared for digestion with endo H as described by Trimble and Maley (1977).

Concanavalin A-binding Assays

Lectin-binding experiments with IPP isomerase were carried out using [^{14}C-*methyl*]concanavalin A (specific activity 13.9 μCi mg^{-1}) and the gel overlay procedure of Chu *et al.* (1981). The gel overlay solution contained 0.02 mg ml^{-1} of the labeled concanavalin A, 5 mg ml^{-1} of hemoglobin in 50 mM Tris-HCl, 600 mM sodium chloride, 0.1 mM calcium chloride, 0.1 mM magnesium chloride, pH 7.5. Samples of IPP isomerase (10 μg) and deoxyribonuclease A (10 μg) were subjected to SDS-PAGE, the gel was stained with Coomassie Blue, equilibrated with Tris-HCl buffer, overlayed with the above solution, and allowed to stand at room temperature for 18 h. Unbound lectin was removed by several washes with Tris-HCl buffer, and the gel was dried onto Whatman No. 3MM paper prior to autoradiography using Kodak X-omat film.

RESULTS

Purification of IPP Isomerase—As part of a study of the mechanism of action of IPP isomerase, we developed an improved procedure for purification of the enzyme from *S. cerevisiae* based on a combination of previous reports (Bruenger *et al.*, 1986; Reardon and Abeles, 1986; Muehlbacher and Poulter, 1988). Our protocol, summarized in Table I, yielded material which gave a single band (>95%) on SDS gels at 39,000 daltons. This molecular weight is consistent with the value of 40,000 found by Reardon and Abeles (1986). However, the specific activity of our preparation was 4.6-fold greater than they reported.

Cloning of the IPP Isomerase Gene—Edman degradation of the purified protein afforded a 35-amino acid sequence in which one position was not assigned and another was ambiguous. The sequence, shown in Fig. 2, was used to construct DNA probes for screening a yeast genomic DNA library for the IPP isomerase gene.

Two probes were constructed from the NH_2-terminal protein sequence (see Fig. 2). Probe 1 was 17 nucleotides in length and degenerate for all possible amino acid codons. Probe 2 was a 56-mer based on codon usage for highly expressed yeast genes (Bennetzen and Hall, 1982). We screened 10,000 plaques of a yeast genomic library for the presence of sequences complimentary to these non-overlapping probes. After plaque purification and rescreening, seven purified phages were obtained. Five contained a 3.5-kb *Eco*RI fragment which bound both probes (Fig. 3). *Eco*RI digestion of the other two phage DNAs resulted in the hybridizing phage DNA region being retained on one arm of the λ vector. The 3.5-kb *Eco*RI fragment was ligated into the phagemid pBluescript SK(+) in both orientations. These constructions were designated pARC133A and pARC133B. Southern blot analysis of these clones revealed both probes hybridized between the *Kpn*I and *Pvu*II sites.

Nucleotide Sequence of IPP Isomerase Gene—Initially, single-stranded DNA from each of the phagemids pARC133A and pARC133B was used as template for sequencing reactions using a subset of the oligonucleotides present in probe 1 as primers. This allowed us to sequence the DNA in the vicinity of the gene corresponding to the NH_2 terminus of the protein and to establish the direction of the gene on the DNA fragment. Sequenced regions were propagated using synthetic primers that hybridized to the ends of previously sequenced

TABLE I

Purification of IPP isomerase from S. cerevisiae

Step	Total units	Total protein	Specific activity	Yield	Purification
	μmol min⁻¹	*mg*	*μmol mg⁻¹*	*%*	*-fold*
pH 5.0 supernatant	130	30,900	0.004	(100)	1
45–75% $(NH_4)_2SO_4$ pellet	115	21,175	0.005	88	1.3
DE52 cellulose	108	772	0.14	83	33
n-Butyl-Sepharose	67	86	0.78	52	186
Chromatofocusing	36	11	3.2	28	762
Sephacryl S-200	33	3.6	9.2	25	2,180
Protein Pak DEAE-5PW	16	1.3	12.3	12	2,930

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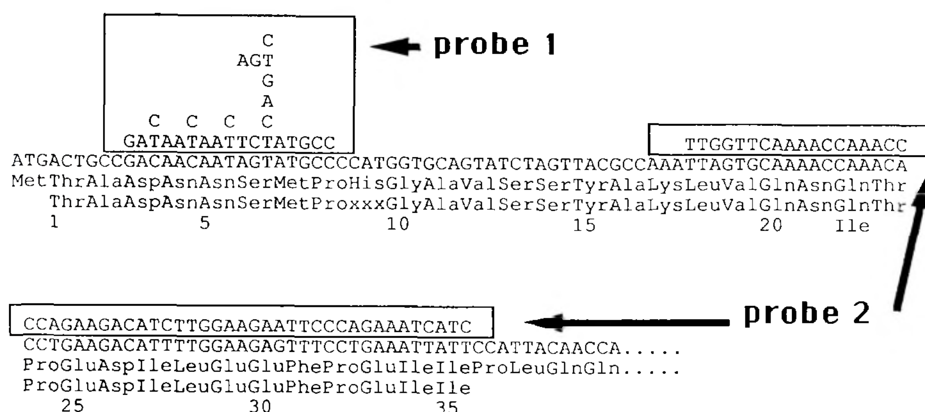

FIG. 2. **Probes used for gene isolation and comparison of protein and DNA sequences for yeast IPP isomerase.** The *top continuous line* is the 5′ sequence of the IPP isomerase gene. Immediately below the DNA sequence is the derived protein sequence. The *bottom line* is the actual protein sequence determined by Edman degradation. Cycle 9 was unassignable, and cycle 22 was ambiguous as either glutamine or isoleucine. Above the DNA sequence are the probes used to screen for the gene. *Probe 1* is a mixture of all possible 17-mers that could code for amino acids 3–8 without the last base of the proline 8 codon. *Probe 2* is a single 56-mer oligonucleotide species that contains only the most commonly used yeast codon for each amino acid.

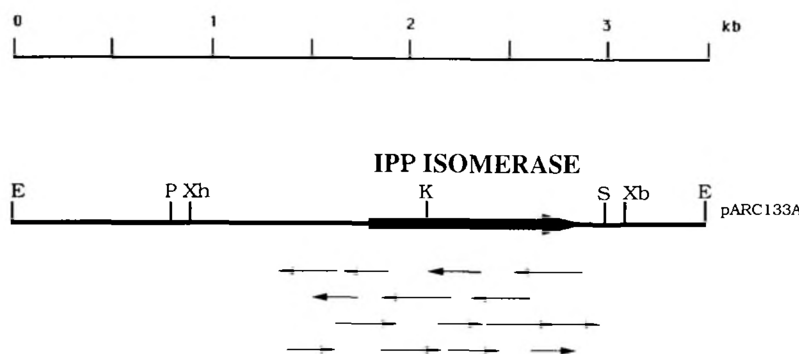

FIG. 3. **Location of IPP isomerase gene on insert of pARC133A.** The 3.5-kb *Eco*RI insert of pARC133A is shown. The *thick arrow* shows IPP isomerase gene location and direction. *Arrows* below show the strategy used for sequencing. pARC133A and pARC133B were used as template for synthetic primers based on probe 1. The DNA sequence in the vicinity of the NH_2 terminus of the protein and the direction of the gene on the DNA fragment were established. Sequenced regions were propagated using synthetic 18-mers designed to hybridize to the ends of previously sequenced DNA. Overlapping sequences *from left to right* were obtained with pARC133A and from right to left with pARC133B. Abbreviations are as follows: *E, Eco*RI; *K, Kpn*I; *P, Pvu*II; *S, Sal*I; *Xb, Xba*I; Xh, *Xho*I.

DNA. The strategy used in sequencing the entire IPP isomerase gene and the restriction map for the region are given in Fig. 3. Both strands were sequenced with overlaps to resolve any ambiguities. The nucleotide sequence of the IPP isomerase gene and flanking DNA is shown in Fig. 4. The structural gene is 864 base pairs and encodes a 33,500-dalton protein.

Fig. 2 shows the DNA sequence of the region complementary to probes 1 and 2. Immediately below the DNA sequence is the amino acid sequence of IPP isomerase derived from the DNA sequence, and on the bottom line is the NH_2-terminal protein sequence determined by Edman degradation. Comparison of the two protein sequences indicates that the native enzyme lacks its initiating methionine. The unassigned amino acid at cycle 9 corresponds to a histidine residue, and the ambiguous signal at cycle 22 in the degradation is glutamine. The perfect match between NH_2-terminal amino acid sequence of the enzyme and the protein sequence derived from the DNA sequence was our first confirmation that our clone contained the IPP isomerase gene. The pI for IPP isomerase of 4.6 predicted from the protein sequence is in good agreement with the value of 4.5 measured by isoelectric focusing (Robertson *et al.*, 1987).

The level of transcription and expression of the IPP isomerase gene can be approximated from the codon bias inherent in the coding sequence. The codon bias index defined by Bennetzen and Hall (1982) measures the extent to which a preferred set of 25 yeast codons of the possible 61 triplets are utilized. The codon bias index has been found to correlate with the protein and mRNA abundance. For IPP isomerase, the codon bias index (see Table III) is low (0.24) and is in the range typically seen for biosynthetic enzymes. Based on the specific activity of purified isomerase, we estimate the enzyme constitutes approximately 0.03% of the soluble protein.

Elevated Expression of the IPP Isomerase Gene—Convincing evidence for the existence of a gene for IPP isomerase in pARC133A was obtained by first subcloning the 3.5-kb *Eco*RI fragment into YRp17 in both orientations, designated pMA150A and pMA150B. Yeast strain JGY195 and transformants containing YRp17, pMA150A, and pMA150B were then assayed for IPP isomerase activity at various times during their growth cycles. The results are summarized in Table II.

In the transformed strains, the specific activity of IPP isomerase reached a maximum in mid-log phase and then decreased by 2–3-fold as cells entered stationary phase. A comparison of peak values of the specific activities (at ap-

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           10        20        30        40        50        60
            |         |         |         |         |         |
   1 GGGTAAATAAATAACAGTCACAGAATAGTATAGGTTATTAAGGCTTCATGCGGCTATTTT
  61 GCCTATATTAATTTCTTTTTTTTTCTTTTTTCTTTTTTTCCGCTGCTATCGGTAAAAGT
 121 TTCCGTTCTTGCTGGAACGAAATGGACAAAACTTAATTGTGATTGAAATTTTTCCAGTCA
 181 ACAGTTTCGCAATGACGATGCCCAGCCGATTCTTTTCCCTTTAATTGTTATTAAGAAACA
 241 TGATTATTCATAACTCTTTTCTTGTCGATGGGGTTGCCTTTCTTTTTCGGTCTTAACTC
 301 CATTTATATTTATTTATTCATTTTTATCTATTTAACAGGAAACAGTTTTCTAGTGACAAG
 361 AAGGCGTATATCCCACTTAATTCAATATTAGAGTATTCGTATTTGGAATACAGGAAGAGT
 421 AAAAATAAGCCAAAAATTCATTACACCTCAATGACTGCCGACAACAATAGTATGCCCCAT
                                     METThrAlaAspAsnAsnSerMETProHis
                                      1                          10
 481 GGTGCAGTATCTAGTTACGCCAAATTAGTGCAAAACCAAACACCTGAAGACATTTTGGAA
     GlyAlaValSerSerTyrAlaLysLeuValGlnAsnGlnThrProGluAspIleLeuGlu
                                     20                          30
 541 GAGTTTCCTGAAATTATTCCATTACAACAAAGACCTAATACCCGATCTAGTGAGACGTCA
     GluPheProGluIleIleProLeuGlnGlnArgProAsnThrArgSerSerGluThrSer
                                     40                          50
 601 AATGACGAAAGCGGAGAAACATGTTTTTCTGGTCATGATGAGGAGCAAATTAAGTTAATG
     AsnAspGluSerGlyGluThrCysPheSerGlyHisAspGluGluGlnIleLysLeuMET
                                     60                          70
 661 AATGAAAATTGTATTGTTTTGGATTGGGACGATAATGCTATTGGTGCCGGTACCAAGAAA
     AsnGluAsnCysIleValLeuAspTrpAspAspAsnAlaIleGlyAlaGlyThrLysLys
                                     80                          90
 721 GTTTGTCATTTAATGGAAAATATTGAAAAGGGTTTACTACATCGTGCATTCTCCGTCTTT
     ValCysHisLeuMETGluAsnIleGluLysGlyLeuLeuHisArgAlaPheSerValPhe
                                    100                         110
 781 ATTTTCAATGAACAAGGTGAATTACTTTTACAACAAAGAGCCACTGAAAAAATAACTTTC
     IlePheAsnGluGlnGlyGluLeuLeuLeuGlnGlnArgAlaThrGluLysIleThrPhe
                                    120                         130
 841 CCTGATCTTTGGACTAACACATGCTGCTCTCATCCACTATGTATTGATGACGAATTAGGT
     ProAspLeuTrpThrAsnThrCysCysSerHisProLeuCysIleAspAspGluLeuGly
                                    140                         150
 901 TTGAAGGGTAAGCTAGACGATAAGATTAAGGGCGCTATTACTGCGGCGGTGAGAAAACTA
     LeuLysGlyLysLeuAspAspLysIleLysGlyAlaIleThrAlaAlaValArgLysLeu
                                    160                         170
 961 GATCATGAATTAGGTATTCCAGAAGATGAAACTAAGACAAGGGGTAAGTTTCACTTTTTA
     AspHisGluLeuGlyIleProGluAspGluThrLysThrArgGlyLysPheHisPheLeu
                                    180                         190
1021 AACAGAATCCATTACATGGCACCAAGCAATGAACCATGGGGTGAACATGAAATTGATTAC
     AsnArgIleHisTyrMETAlaProSerAsnGluProTrpGlyGluHisGluIleAspTyr
                                    200                         210
1081 ATCCTATTTTATAAGATCAACGCTAAAGAAAACTTGACTGTCAACCCAAACGTCAATGAA
     IleLeuPheTyrLysIleAsnAlaLysGluAsnLeuThrValAsnProAsnValAsnGlu
                                    220                         230
1141 GTTAGAGACTTCAAATGGGTTTCACCAAATGATTTGAAAACTATGTTTGCTGACCCAAGT
     ValArgAspPheLysTrpValSerProAsnAspLeuLysThrMETPheAlaAspProSer
                                    240                         250
1201 TACAAGTTTACGCCTTGGTTTAAGATTATTTGCGAGAATTACTTATTCAACTGGTGGCAG
     TyrLysPheThrProTrpPheLysIleIleCysGluAsnTyrLeuPheAsnTrpTrpGlu
                                    260                         270
1261 CAATTAGATGACCTTTCTGAAGTGGAAAATGACAGGCAAATTCATAGAATGCTATAACAA
     GlnLeuAspAspLeuSerGluValGluAsnAspArgGlnIleHisArgMETLeu---
                                    280
1321 CGCGTCAATAATATAGGCTACATAAAAATCATAATAACTTTGTTATCATAGCAAAATGTG
1381 ATATAAAACGTTTCATTTCACCTGAAAAATAGTAAAAATAGGCGACAAAAATCCTTAGTA
1441 ATATGTAAACTTTATTTTCTTTATTTATTTACAGAACTCTGAATATACATTGATTGTTCA
1501 CATTTTT
```

FIG. 4. **Nucleotide and presumptive amino acid sequence of the IPP isomerase gene.** Potential TATA and termination signals are *underlined*. A large poly(dA·dT) region at the 5′ end of the gene is also *underlined*.

TABLE II

Inhibition of IPP isomerase by 3-(fluoromethyl)-3-buten-1-yl disphosphate (FIPP) and 3,4-epoxy-1-butenyl diphosphate (EBPP)

Strain	Specific activity[a]		
	Control[b]	FIPP (% inhibition)[c]	EBPP (% inhibition)[d]
	milliunits mg^{-1}		
JGY195	4	0.2 (95)	0.3 (93)
JGY195/YRp17	8	0.2 (98)	1.0 (88)
JGY195/pMA150A	47	5.9 (87)	2.5 (95)
JGY195/pMA150B	43	4.3 (90)	1.5 (97)
IPP isomerase[e]	122	7.9 (94)	13.4 (89)

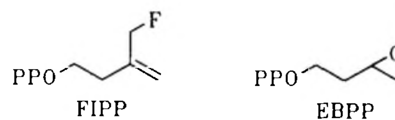
[a] Determined for cell-free extracts from cultures after 18 h of incubation. Transformed strains were at mid-log phase. Untransformed JGY195 was in late-log to early stationary phase.

[b] Preincubated for 20 min at 37 °C before addition of IPP.

[c] Preincubated with 10 μM FIPP for 20 min at 37 °C before addition of IPP.

[d] Preincubated with 100 μM EBPP for 20 min at 37 °C before addition of IPP.

[e] Partially purified from *S. cerevisiae*.



SCHEME 2

TABLE III

Codon usage in the IPP isomerase structural gene

AA, amino acid.

AA	Codon	No.	AA	Codon	No.	AA	Codon	No.	AA	Codon	No.
Phe	TTT	9	Ser	TCT	5	Tyr	TAT	1	Cys	TGT	4
	TTC	5		TCC	1		TAC	5		TGC	3
Leu	TTA	12		TCA	2		TAA	1		TGA	0
	TTG	5		TCG	0		TAG	0	Trp	TGG	7
Leu	CTT	3	Pro	CCT	5	His	CAT	9	Arg	CGT	1
	CTC	0		CCC	1		CAC	1		CGC	0
	CTA	6		CCA	8	Gln	CAA	10		CGA	1
	CTG	0		CCG	0		CAG	0		CGG	0
Ile	ATT	16	Thr	ACT	8	Asn	AAT	13	Ser	AGT	4
	ATC	3		ACC	2		AAC	9		AGC	2
	ATA	1		ACA	4	Lys	AAA	7	Arg	AGA	6
Met	ATG	7		ACG	2		AAG	12		AGG	2
Val	GTT	4	Ala	GCT	4	Asp	GAT	11	Gly	GGT	11
	GTC	3		GCC	4		GAC	10		GGC	1
	GTA	1		GCA	3	Glu	GAA	2		GGA	1
	GTG	3		GCG	2		GAG	6		GGG	0

proximately 15 h) revealed that transformants containing the plasmids pMA150A or pMA150B were 5–6-fold higher than in the transformant containing YRp17. The nearly equal levels of IPP isomerase activity in JGY195/pMA150A and JGY195/pMA150B indicate that expression is independent of orientation. This is an expected result since the 3.5-kb *Eco*RI fragment contains ample regions flanking the promotor and terminator elements of the structural gene.

Specific active site-directed irreversible inhibitors of IPP isomerase were used to establish that the elevated specific activities measured in crude extracts could be attributed directly to the enzyme. Cell-free extracts of a late-log culture of JGY195 and mid-log cultures of JGY195/YRp17, JGY195/pMA150A, and JGY195/pMA150B were preincubated with 3-(fluoromethyl)-3-buten-1-yl diphosphate or 3,4-epoxy-1-butenyl diphosphate. These compounds are known to act as potent, specific inhibitors of IPP isomerase (Muehlbacher and Poulter, 1988; Muehlbacher *et al.*, 1989) (Scheme 2). The results of these experiments are summarized in Table III. In each case, preincubation with 3,4-epoxy-1-butenyl diphosphate or 3-(fluoromethyl)-3-buten-1-yl diphosphate drastically lowered the specific activity of IPP isomerase. The percentage of inhibition was 87–98% for all strains.

Tests for Post-translational Modification—The discrepancy between the molecular masses of 33,350 daltons deduced from the gene sequence of IPP isomerase and 39,000–40,000 daltons determined by SDS-PAGE suggest that the protein is modified post-translationally, perhaps by glycosylation. The amino acid sequence contains potential glycosylation sites at Asn-5, Asn-22, and Asn-221. Since the amino-terminal Edman degradation spanned positions 5 and 22, only Asn-221 is a likely candidate.

Digestion of IPP isomerase with endo H did not change the apparent molecular weight of the polypeptide as deduced by comparison with an untreated control on SDS-PAGE under conditions where we easily detected a loss of approximately 2000 daltons from deoxyribonuclease A when treated under similar conditions (Tarentino *et al.*, 1974). In addition, no change was seen for IPP isomerase when the enzyme was

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denatured with SDS prior to endo H treatment (Chu *et al.*, 1981).

Binding experiments between IPP isomerase and concanavalin A also gave negative results. Samples of IPP isomerase and deoxyribonuclease A were subjected to SDS-PAGE. The gel was treated with ^{14}C-methylated concanavalin A, and nonspecifically bound lectin was removed by repeated washing. Autoradiography showed that ^{14}C-labeled concanavalin A only bound to deoxyribonuclease A.

DISCUSSION

By combining features of several purifications (Bruenger *et al.*, 1986; Abeles and Reardon, 1986; Muehlbacher and Poulter, 1988), we obtained a preparation of IPP isomerase from *S. cerevisiae* with a 4–5-fold higher specific activity than previously reported (Reardon and Abeles, 1986). The IPP isomerase gene was then isolated from a λ library of yeast genomic DNA using oligonucleotide probes whose sequences were based on the NH_2-terminal sequence of the enzyme. A 3.5-kb *Eco*RI fragment was obtained that encoded a protein of 288 amino acids with a molecular mass of 33,350 daltons. The identity of the gene, which we have designated *bot 2* (biosynthesis of terpenes), was established by subcloning the 3.5-kb *Eco*RI fragment into a yeast shuttle vector which produced enhanced levels of IPP isomerase activity in yeast transformants.

There is a significant discrepancy between the molecular mass of IPP isomerase of 33,350 daltons deduced from the gene sequence and the values of 39,000–40,000 daltons determined by SDS-PAGE of the purified enzyme by us and by Reardon and Abeles (1986). We initially suspected that IPP isomerase might be glycosylated, and inspection of the amino acid sequence suggested potential glycosylation sites at asparagines 5, 22, and 221. However, Edman degradation of the amino terminus ruled out glycosylation at Asn-5 and Asn-22. Attempts to detect glycosyl units at other sites were unsuccessful. The mobility of IPP isomerase upon SDS-PAGE did not change after treatment with endo H. This enzyme hydrolyzes glycoproteins at the di-*N*-acetylchitobiosyl core common to most *N*-linked glycoproteins (Tarentino and Maley, 1974; Tarentino *et al.*, 1974) with a resulting decrease in molecular weight of the polypeptide. Since the susceptibility to endo H hydrolysis can vary, we also attempted endo H hydrolysis of denatured IPP isomerase (Chu *et al.*, 1981) without success. In addition, treatment of the enzyme with radiolabeled concanavalin A did not reveal detectable levels of binding between IPP isomerase and the lectin, suggesting the absence of α-glucoside or α-mannoside linkages (Osawa and Tsuji, 1987). The absence of positive indications upon treatment with endo H or concanavalin A and the fact that IPP isomerase is a cytosolic enzyme suggest that the protein is not glycosylated. At this point, the discrepancy between molecular weights obtained from the gene sequence and SDS-PAGE of the enzyme remains unexplained.

Similar absolute levels of expression were observed in JGY195/pMA150A and JGY195/pMA150B. In addition, similar patterns of variation of enzyme activity during cell growth were observed with both clones. This suggests, as expected from the large 5′-flanking region present, that IPP isomerase expression is being controlled by elements flanking the gene and not by vector sequences. No consensus TATA box sequence (TATAAA) is found upstream of the IPP isomerase coding region. A sequence differing by one base from the consensus is located 220 base pairs upstream from the initial ATG codon and is *underlined* in Fig. 4. Another feature of the upstream sequence is the long stretch of poly(dA·dT), also *underlined*. Stretches of poly(dA·dT) serve as upstream promotor elements for other yeast genes (Struhl, 1985). A possible transcription termination sequence (Zaret and Sherman, 1982) is also underlined in Fig. 4.

In actively growing cultures of JGY195 transformed with pMA150A and pMA150B, the specific activity of IPP isomerase peaked during mid-log phase and then declined by 2–2.5-fold as the cultures moved into stationary phase. These results are in contrast to levels for the next enzyme in the pathway, farnesyl diphosphate synthetase, where the specific activity of the enzyme remains constant in controls and transformants containing multiple copies of the farnesyl diphosphate synthetase gene (Anderson *et al.*, 1989). DMAPP is the ultimate source of allylic substrates needed to prime the prenyltransferases for chain elongation by condensation with IPP. Thus, the relative demand for DMAPP is higher during periods of maximal sterol biosynthesis, which utilizes farnesyl diphosphate without further chain elongation, than during biosynthesis of higher prenyl diphosphates needed for production of dolichols and ubiquinones where additional IPP is required. If differential needs exist for isoprenoids of different chain lengths, control of the activity of IPP isomerase might provide a mechanism for regulating the relative levels of IPP and DMAPP to match metabolic requirements.

IPP isomerase catalyzes an essential activation step in isoprenoid metabolism in the conversion of IPP to DMAPP by enhancing the electrophilicity of the isoprene unit by at least a billion-fold. There are no known IPP isomerase mutants for any organism, and until recently (Bruenger *et al.*, 1986; Reardon and Abeles, 1986; Dogbo and Camara, 1987; Muehlbacher and Poulter, 1988), no reliable purifications of the enzyme.[2]

The reaction catalyzed by IPP isomerase is a simple [1.3] hydrogen migration. During the isomerization of IPP to DMAPP, the added proton is ultimately derived from water, presumably by exchange with an enzymic acid, and the released proton is lost to water. The reaction is antarafacial (Clifford *et al.*, 1971). Thus, two bases, one in the conjugate acid form, are required for catalysis. Studies with alternate substrates and transition state analogues (Poulter and Muehlbacher, 1988; Abeles and Reardon, 1986) established that the mechanism for isomerization is a protonation-deprotonation reaction via a tertiary carbocation. Enzymatic protonations of unactivated olefins are rarely encountered in nature. The only prominent examples are IPP isomerase, Δ^8–Δ^7 cholesterol isomerase (Wilton *et al.*, 1969), and enzymes catalyzing proton-initiated cyclizations of isoprenoids (Goodwin, 1981). There are no active site structures for any of the members in this group and how catalysis is achieved is a mystery. We recently discovered a family of suicide substrates and highly specific active site-directed irreversible inhibitors for isomerase (Muehlbacher and Poulter, 1988; Muehlbacher *et al.*, 1989). Although the site of covalent attachment is presently unknown, knowledge of the complete amino acid sequence of isomerase will facilitate identification of the location of residues in the catalytic site.

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[2] Attempts in our laboratory (C. D. P.) and by Rilling (private communication) to purify porcine liver IPP isomerase by the Banthorpe procedure (Banthorpe *et al.*, 1977) were not successful.

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Addendum—We have succeeded in expressing the gene for yeast IPP isomerase in *E. coli.* The purified, active enzyme behaved identically to that obtained from yeast upon SDS-gel electrophoresis. We conclude that the difference in molecular weights predicted from the gene sequence and from SDS-gels is not due to glycosylation.

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