Yeast Protein Farnesyltransferase. Site-Directed Mutagenesis of Conserved Residues in the β-Subunit†

Julia M. Dolence, David B. Rozema,‡ and C. Dale Poulter*

Department of Chemistry, University of Utah, Salt Lake City, Utah 84112

Received January 13, 1997; Revised Manuscript Received May 16, 1997⊗

ABSTRACT: Protein prenyltransferases catalyze the posttranslational modification of cysteines by isoprenoid hydrocarbon chains. A protein farnesyltransferase (PFTase) and a protein geranylgeranyltransferase (PGGTase-I) alkylate cysteines in a CaaX C-terminal tetrapeptide sequence, where a is usually an aliphatic amino acid and X is an amino acid that specifies whether a C_{15} farnesyl or C_{20} geranylgeranyl moiety is added. A third enzyme, PGGTase-II, adds geranylgeranyl groups to both cysteines at the C-terminus of Rab proteins. All three enzymes are Zn^{2+} metalloproteins and also require Mg^{2+} for activity. The protein prenyltransferases are heterodimers. PFTase and PGGTase I contain identical α-subunits and distinctive β-subunits, which are responsible for the differences in substrate selectivity seen for the two enzymes. The subunits in PGGTase-II are similar, but not identical, to their counterparts in the other two enzymes. An alignment of amino acid sequences for the β-subunits of all three enzymes shows five regions of high similarity. Thirteen of the conserved polar and charged residues in yeast PFTase were selected for substitution by site-directed mutagenesis. Kinetic studies revealed a subset of five enzymes, R211Q, D307A, C309A, Y310F, and H363A, with substantially reduced catalytic constants (k_{cat}). Metal analyses of wild-type enzyme and the five least reactive mutants showed that the substitutions had compromised Zn^{2+} binding in the D307A, C309A, and H363A enzymes.

Protein prenyltransferases catalyze the transfer of alkyl groups from allylic prenyl diphosphates to cysteine residues at the C-terminus of a wide variety of proteins in eukaryotic organisms. The prenylated proteins include "true" Ras proteins, other members of the Ras superfamily (Rap, Rab, Rac, Ral), large G proteins, nuclear lamins, and phosphodiesterases (Clarke, 1992). Protein farnesyltransferase (PFTase)[1] transfers the C_{15} farnesyl group to proteins containing C-terminal CaaX recognition sequences where C is cysteine, "a" is an aliphatic amino acid, and "X" is Ser, Ala, Cys, Met, or Gln (Moores et al., 1991; Reiss et al., 1991; Omer et al., 1993). PFTase was initially purified from rat brain (Reiss et al., 1990a,b) and subsequently from bovine brain (Pompliano et al., 1992a) using CaaX-based peptide affinity columns. Recombinant yeast (Mayer et al., 1993) and human (Omer et al., 1993) PFTases were purified by immunoaffinity chromatography. Steady-state and pre-steady-state kinetic studies indicated an ordered mechanism with FPP binding first (Pompliano et al., 1993; Furfine et al., 1995; Dolence et al., 1995; Mathis & Poulter, 1997). A related enzyme, protein geranylgeranyltransferase-I (PGGTase-I), modifies CaaX-containing proteins where "X" is Leu or Phe with a C_{20} geranylgeranyl group (Yokoyama et al., 1991; Moores et al., 1991; Caplin et al., 1994). A third enzyme, PGGTase-II, alkylates both cysteines in Rab proteins with C-terminal -CC or -CXC sequences (Horiuchi et al., 1991; Moores et al., 1991; Seabra et al., 1992).

The protein prenyltransferases are heterodimers. PFTase and PGGTase-I have the unusual feature of containing identical α-subunits in combination with distinctive β-subunits (Armstrong et al., 1993). Thus, the factors that lead to different substrate selectivities for the two enzymes must reside in their β-subunits. PGGTase-II has the additional feature of requiring a "Rab escort protein" (REP), to present the protein substrate to the catalytic heterodimer and to remove the geranylgeranylated product. PFTase and PGGTase-I recognize short peptides as substrates, provided that they contain a C-terminal CaaX sequence. In contrast, PGGTase-II cannot utilize small peptide substrates and apparently requires an additional recognition motif in the full protein.

All three protein prenyltransferases are Zn^{2+} metalloenzymes that require the divalent metal for activity. A quantitative metal analysis for PFTase indicates that the enzyme contains one atom of Zn^{2+} per 1 mol of heterodimer (Chen et al., 1993). Metal-depleted PFTase and PGGTase-I retain their ability to bind prenyl substrates (Reiss et al., 1992; Yokoyama et al., 1995). However, PFTase does not bind its protein substrates in the absence of Zn^{2+} (Reiss et al., 1992). Mammalian PGGTase-II was reported to be strongly inhibited by Zn^{2+} (Seabra et al., 1992). However, Witter and Poulter (1996) found that the activity of Zn^{2+}-depleted yeast PGGTase-II was stimulated by addition of low concentrations of Zn^{2+} and inhibited at higher concentrations, a phenomenon encountered with some other prenyltransferases (Sagami et al., 1984). Zn^{2+} facilitates binding of the peptide substrates and may enhance the nucleophilicity

† This work was supported by National Institutes of Health Grant GM 21328.

* To whom correspondence should be addressed.

‡ National Science Foundation Postdoctoral Fellow.

⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1997.

[1] Abbreviations: BME, 2-mercaptoethanol; CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPP, farnesyl diphosphate; Pep, dansyl-GCVIA; PMSF, phenylmethanesulfonyl fluoride; PFTase, protein farnesyltransferase; PGGTase, protein geranylgeranyltransferase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; wt, wild type.

S0006-2960(97)00039-1 CCC: $14.00

of the sulfhydryl group during prenyl transfer. Like most prenyltransferases, all three protein prenyltransferases require Mg^{2+} for catalytic activity (Moomaw & Casey, 1992; Reiss et al., 1992; Chen et al., 1993; Seabra et al., 1992).

Recently, several groups have studied mutant forms of the enzymes in an effort to identify amino acids that play important roles in binding and catalysis. Random mutagenesis of yeast PFTase was used to identify mutants that acquired the substrate selectivity of PGGTase-I (Mitsuzawa et al., 1995). One of these proteins, the S159N β-subunit mutant, showed a 20-fold increase in its utilization of peptide substrates for PGGTase-I and a 6-fold decrease for farnesylation of peptide substrates for PFTase. Several deletion and point mutations were also reported in the α-subunit of PFTase. Normal activity was observed when 51 amino acids were deleted from the N-terminus, while deletion of 106 amino acids abolished activity (Andres et al., 1993). In contrast, deletion of only 5 residues at the carboxy terminus reduced activity appreciably. Substitution of asparagine for a conserved lysine at position 164 produced an enzyme whose activity was significantly reduced, but substrate binding and subunit association were not affected. Mutations of other conserved residues in the α-subunit (Y166F, R172E, N199D, W203H, and N199K) affected PFTase activity to varying degrees (Andres et al., 1993; Omer et al., 1993). Three site-directed mutations (D200N, G249V, and G349S) were made in the β-subunit of human PFTase at locations corresponding to those identified in the yeast enzyme (Omer et al., 1993). The G249V mutation resulted in a decrease in the affinity of both substrates, while the other two mutants showed only a decrease in the affinity of the protein substrate.

Although Zn^{2+} is clearly a crucial component of the catalytic machinery for protein prenyltransferases, the role of the metal in catalysis is not known. It could serve to bind and enhance the nucleophilicity of the cysteine sulfhydryl in the protein substrate, to bind and enhance the reactivity of the diphosphate moiety in the isoprenoid substrate, or to serve in a structural role not involving a direct interaction of either substrate. Recently Fu et al. (1996) identified Cys^{299} in the β-subunit of rat PFTase as an active-site residue that was only modified by *N*-ethylmalemide after Zn^{2+} had been removed from the enzyme. They constructed C299A and C299S mutants and found that the proteins were inactive catalysts which had also lost their ability to bind Zn^{2+}. We have constructed several β-subunit mutants corresponding to conserved polar residues in yeast PFTase that were identified by aligning amino acid sequences for the subunits of PFTases, PGGTases-I, and PGGTases-II from a variety of sources. We now report the results of studies with 13 mutants, which include several proteins with altered catalytic activity and Zn^{2+} binding properties.

MATERIALS AND METHODS

Materials. Farnesyl diphosphate (FPP) was synthesized by the method of Davisson et al. (1986). Dansyl-GCVIA (Pep) and DNA primers for site-directed mutagenesis were prepared at the Core facility for DNA and peptide synthesis at the University of Utah. *n*-Dodecyl β-D-maltoside was purchased from CalBiochem. Unless specified, all other materials were purchased from Sigma (St. Louis, MO).

Methods. All protein purification steps were conducted at 4 °C, and eluted proteins were detected at 280 nm. After the final purification step, fractions were analyzed by 12% stacking SDS−PAGE after staining with Coomassie Blue R. Protein concentrations were measured by the method of Bradford (1976) with bovine serum albumin as the standard. Site-directed mutants were prepared using the Mutagene kit (BioRad) or the Morph Site-Specific Plasmid DNA Mutagenesis kit (5 Prime → 3 Prime, Inc.). DNA sequencing was performed at the Core Sequencing Facility (University of Utah). Sequence comparisons were performed with the Clustal Program of the PC/GENE Computer package (Intelligenetics, Inc.). Metal ion analyses were performed by Chemical Analysis Laboratory (Athens, GA).

Site-Directed Mutagenesis. For mutants H156A and H363A, site-directed mutagenesis was performed on the *RAM*1 open reading frame in pGP14 (Mayer et al., 1993) using a repair-deficient host with the Morph kit (5 Prime → 3 Prime, Inc.). For all other mutants, site-directed mutagenesis was performed using Kunkel mutagenesis (1985) on single-stranded uracil-containing DNA obtained from pGP14. Mutagenic primers (31−55 bases) contained mismatches to change the desired residue and to generate a silent mutation that introduced or eliminated a diagnostic restriction site. All mutations were confirmed by DNA sequencing.

Prenyltransferase Assays. Catalytic rate constants (k_{cat}) were measured using a fluorescence assay that continuously monitored farnesylation of Pep (Pompliano et al., 1992b; Cassidy et al., 1995) using a Spex FluoroMax Model spectrofluorometer with $\lambda_{ex} = 340$ nm (slit width = 5.1 or 8.5 nm) and $\lambda_{em} = 486$ nm (slit width = 5.1 or 8.5 nm) and 3 mm square cuvettes. Assays (250 μL) were conducted at 30 °C in 50 mM Tris·HCl, 10 mM $MgCl_2$, 10 μM $ZnCl_2$, 5 mM DTT, and 0.04% (w/v) *n*-dodecyl β-D-maltoside, pH 7.0. PFTase was added to initiate the reactions. Due to substrate inhibition (Dolence et al., 1995), a saturating concentration of the peptide substrate was not used to measure K_M^{FPP}. Instead, a concentration that gave a maximal rate for wild-type (wt) PFTase (2.4 μM) was chosen unless otherwise specified. The saturating FPP concentration used for V_{Max} and K_M^{Pep} determinations was 150 μM, sufficient to overcome substrate inhibition. Rates were measured from the linear region of each run, and all measurements were made in duplicate. Rates were measured in counts per second per second and converted to units of micromolar per second using a conversion factor (*m*) calculated from the slope of a line generated in a plot of concentration of synthetic dansyl-G(S-farnesyl)CVIA versus fluorescence intensity (Cassidy et al., 1995).

Protein Synthesis and Purification. *Escherichia coli* XA90 was transformed with pGP14 or related plasmids containing the site-directed mutations (see Table 1). Overnight cultures of single colonies in Luria−Bertani medium containing 100 μg of ampicillin/mL were used to inoculate 500 mL of super broth (32 g of bactotryptone/20 g of yeast extract/5 g of NaCl/5 mL of 1 M NaOH/50 mg of Amp, all per liter). Cells were harvested after 12−16 h at 37 °C, 250 rpm. Wet cells were frozen at −80 °C.

Frozen cell paste from a 500 mL incubation was thawed on ice. The cells were resuspended in 50 mL of sonication buffer (50 mM Tris·HCl, pH 7.0, 5 mM $MgCl_2$, 10 mM BME, and 1 mM PMSF). The suspension was disrupted by sonication and clarified by centrifugation. The cell-free supernatant was loaded onto a 1.5 × 15 cm DE-52 (Whatman) column preequilibrated with 50 mM Tris·HCl, pH 7.0,

5 mM $MgCl_2$, 50 μM $ZnCl_2$, and 10 mM BME (DE52 buffer). After the absorbance at 280 nm returned to base line, the column was eluted with a stepwise gradient of DE52-buffer containing 100 mM NaCl, 200 mM NaCl, and 1 M NaCl. During the 200 mM NaCl elution step, the first 15 mL was discarded, and the next 35−45 mL was collected and saved for purification by immunoaffinity chromatography on an anti-α-tubulin column. For the H156A and H363A mutants, $ZnCl_2$ was omitted from the sonication and DE52 buffers.

DE52-purified material was loaded at 0.1 mL/min onto the antibody column (Mayer et al., 1993), which had been equilibrated with 10 mM sodium phosphate, pH 7.0, 150 mM NaCl, and 10 mM BME (binding buffer). After the column was loaded, it was washed with binding buffer until the absorbance at 280 nm returned to base line. The column was eluted with binding buffer containing 5 mM Asp-Phe. The protein peak was collected as a single 3−5 mL fraction.

Enzyme from the affinity step was diluted with an equal volume of 50 mM Tris·HCl, pH 7.0, 5 mM $MgCl_2$, and 10 mM BME (buffer A) and loaded onto a Poros HQ column (4.6 × 50 mm) which had previously been equilibrated with buffer A at 0.4 mL/min using a Pharmacia FPLC system. The column was washed with a linear gradient of buffer A to 40% buffer B (50 mM Tris·HCl, pH 7.0, 5 mM $MgCl_2$, 1 M NaCl, and 10 mM BME) over 40 mL. PFTase eluted between 28 and 32% buffer B. Fractions were examined for purity by SDS−PAGE. PFTase was typically 90−95% pure. Samples were stored at −80°C in 33% glycerol or on ice at 4 °C.

Metal Ion Analysis. Immunoaffinity-purified wt PFTase and the R211Q, D307A, C309A, Y310F, and H363A mutants were further purified by chromatography on a 4.6 × 100 mm Pharmacia Source Q column that had been equilibrated with 50 mM Tris·HCl, pH 7.0, and 5 mM DTT at 8.5 mL/min using a BioCAD Workstation perfusion chromatographic system (PerSeptive Biosystems, Inc.). The column was washed with a linear gradient from 50 mM Tris·HCl, pH 7.0, and 5 mM DTT to 40% 50 mM Tris·HCl, pH 7.0, 1 M NaCl, and 5 mM DTT over 25 column volumes. PFTase eluted between 0.28 and 0.30 M NaCl. Samples were analyzed by SDS−PAGE.

The enzymes (2 mL total volume) were placed in dialysis bags (12−14 kDa cutoff, Spectrapor) that had been soaked for 6 h in 800 mL of deionized water and dialyzed at 4 °C for 24 h against 1 L of 50 mM Tris·HCl, pH 7.0, 10 mM BME, and 5 mM EDTA. The samples were then dialyzed against 1 L of 50 mM Tris·HCl, pH 7.0, and 10 mM BME that had been passed through a 2.5 × 20 cm column of Chelex 100 resin (BioRad). Chelex 100 resin (25 mL) was also added to the polystyrene dialysis container. Protein concentrations were determined (Bradford, 1976) before the samples were submitted for analysis.

Zn^{2+} Depletion Studies. Samples of wt PFTase and the H156A mutant were dialyzed against 500 mL of 50 mM Tris·HCl, pH 7.0, 10 mM BME and stored on ice. The dialyzed enzymes (4−7 μg) were diluted 20-fold into 50 mM Tris·HCl, pH 7.0, 25 mM EDTA, 2 mM 1,10-phenanthroline, and 5 mM DTT. Samples containing 100−180 ng of enzyme were removed periodically and assayed for activity as described above except Zn^{2+} was omitted from the assay buffer. The Zn^{2+} metalloenzymes were reconstituted by incubation in assay buffer.

CD Measurements. CD measurements were acquired on an AVIV Model 62DS spectrometer. PFTase and mutant protein samples were dialyzed against 2 × 1 L of 10 mM Tris·HCl, pH 7.0, 1 mM BME. For CD measurements of metal-free PFTase and mutants, enzyme samples were first dialyzed against 500 mL of 50 mM Tris·HCl, pH 7.0, 10 mM EDTA, 2 mM 1,10-phenanthroline, and 5 mM BME and then against 2 × 1 L of 10 mM Tris·HCl, pH 7.0, 1 mM BME. Samples (300 μL) of 0.05−0.18 mg/mL were analyzed from 250 to 190 nm in 1.0 nm increments. Final spectra were obtained by signal averaging 10 scans. Measurements were corrected for background by subtracting the CD spectra of 10 mM Tris·HCl, pH 7.0, 1 mM BME. Protein concentrations were determined by measuring the UV absorption at 280 nm using a calculated extinction coefficient of $\epsilon = 1.999$ mL mg^{-1} cm^{-1} (153 340 M^{-1} cm^{-1}) based on the number of tryptophan, tyrosine, and cysteine residues in the heterodimer (Gill & von Hippel, 1989). All spectra were normalized to the concentration of wt PFTase. Data smoothing was performed using GRAFIT (Erithicus Software, Staines, U.K.) using four iterations of the smoothing function.

RESULTS

Sequence Alignments. The CLUSTAL program (Higgins & Sharp, 1988) from PC/GENE was used to align amino acid sequences for the β-subunits of PFTase from humans (Omer et al., 1993), rats (Chen et al., 1991), cows (Omer et al., 1993), and yeast (Goodman et al., 1988); PGGTase-I from humans (Zhang et al., 1994), rats (Zhang et al., 1994), and yeast (Ohya et al., 1991); and PGGTase-II from rats (Armstrong et al., 1993) and yeast (Rossi et al., 1991). The alignments revealed several regions with a high degree of sequence similarity that contained conserved residues which might be important for catalytic activity. We selected 13 polar or charged residues (shown in boldface) from the set of similar and conserved amino acids shown in Figure 1 for substitution by site-directed mutagenesis.

Production and Purification of Site-Directed Mutants of Yeast PFTase. Mutants of yeast PFTase were constructed from pGP14, a plasmid used for coupled heterologous expression of the *RAM1/RAM2* genes in *E. coli* (Mayer et al., 1993). This system produces yeast PFTase containing a Glu-Glu-Phe α-tubulin epitope on the C-terminus of the β-subunit (Ram1) of the enzyme to facilitate purification by immunoaffinity chromatography on a YL1/2 antibody column. The Kunkel (1985) procedure or the Morph kit (5 Prime → 3 Prime, Inc.) was used to create the site-directed mutations in Ram1 shown in Table 1. Silent mutations were used to introduce or eliminate diagnostic restriction sites in order to facilitate identification of the mutants by restriction analysis. All of the new constructs were sequenced in the region of the mutations to confirm their structures.

The mutant proteins, except those containing E256A and R301Q substitutions, accumulated to similar levels seen for the recombinant wild-type enzyme. These two mutants were obtained in somewhat smaller amounts. Cells were disrupted by sonication, and the homogenates were spun to remove cellular debris. The clarified supernatants were purified in three steps. Proteins were first eluted from a DE52 column using a NaCl step gradient. The material collected in the 200 mM NaCl step was loaded onto an α-tubulin column

```
Yeast PFTase    142PSGGPFGG-------GPG-----QLSHLAS159   191SLKEPN-GGFKTCLE--------VGEVDTRGI213
Human PFTase    135SPEGGFGG-------GPG-----QYPHLAP152   183SLKQPD-GSFLM-HV--------GGEVDVRSA204
Rat PFTase      135SPDGGFGG-------GPG-----QYPHLAP152   183SLKQPD-GSFLM-HV--------GGEVDVRSA204
Bovine PFTase   135SPEGGFGG-------GPG-----QYPHLAP152   183SLKQPD-GSFLM-HD--`------GGEVDVRSA204
Pea PFTase       80DPNGGYAG-------GPG-----QMPHLAT 97   128RMKQPN-GGFRM-HD--------EGEIDVRAC149
Human PGGTaseI   95FRGSSYLGIPFNPSKAPGTAHPYDSGHIAM124   153ALQLED-GSFCAVPE--------GSENDMRFV175
Rat PGGTaseI     95FRGSSYLGIPFNPSKNPGTAHPYDSGHIAM124   153ALQLED-GSFCAVPE--------GSENDMRFV175
Yeast PGGTaseI   87FVGSLVMNIPHATTI-----------NLPN105   137KCQRPDRGSFVSCLDYKTNCGSSVDSDDLRFC168
Pombe PGGTaseI   82RTG-INPISFEQE------------PQLAG 99   128LCKTSQG-------HFRSIAVPSCSDQDMRQL152
Yeast PGGTaseII  72-----YGAFAPFPRHDA---------HLLT 87   118GNQLED-GSFQ---------GDRFGEVDTRFV139
Rat PGGTaseII    82-----CGGVSASIGHDP---------HLLY 97   125SLQKED-GSFA---------GDIWGEIDTRFS146
                           .                  ..            .  .                  .. * *

Yeast PFTase    237YLKNCQNYEGGFGSCPHVDEAHGGYT262       293QEERGFCGRSNKLVDGCYSFWVGGS317
Human PFTase    228WIARCQNWEGGIGGVPGM-EAHGGYT252       283RFEGGFQGRCNKLVDGCYSFWQAGL307
Rat PFTase      228WIARCQNWEGGIGGVPGM-EAHGGYT252       283RFEGGFQGRCNKLVDGCYSFWQAGL307
Bovine PFTase   228WIARCQNWEGGIGGVPGM-EAHGGYT252       283RFEGGFQGRCNKLVDGCYSFWQAGL307
Pea PFTase      173FILSCQTYEGGLAGEPGS-EAHGGYT197       228K-ECGFQGRTNKLVDGCYSFWQGGA251
Human PGGTaseI  199YIRRSMSYDNGLAQGAGL-ESHGGST223      255RQQNGYHGRPNKPVDTCYSFWVGAT279
Rat PGGTaseI    199YIRRSMSYDNGLAQGAGL-ESHGGST223      255RQQNGYHGRPNKPVDTCYSFWVGAT279
Yeast PGGTaseI  198YIMSQQCYNGAFGAH---NEPHSGYT220      272SDDGGFQGRENKFADTCYAFWCLNS296
Pombe PGGTaseI  175YIMSCQRYEGGFSLLP-YGEAHAGAT199      249---GGLNGRTNKDVDTCYAYWVLSS270
Yeast PGGTaseII 163FVLKCYNFDGGFGLCPN-AESHAAQA187      222--EGGLNGRPSKLPDVCYSWWVLSS244
Rat PGGTaseII   170FVLSCMNFDGGFGCRPG-SESHAGQI194      226--SGGLNGRPEKLPDVCYSWWVLAS248
                   .. .    ......     *.*..                 *  ** .*  * **..*  .

Yeast PFTase    342CCQEKEQPGLRDKPGAHSDFYHTNYCLL369
Human PFTase    342CCQCPAG-GLLDKPGKSRDFYHTCYCLS368
Rat PFTase      342CCQCPAG-GLLDKPGKSRDFYHTCYCLS368
Bovine PFTase   342CCQCPTG-GLLDKPGKSRDFYHTCYCLS368
Pea PFTase      339CSQEQDG-GLRDKPGKRRDHYHSCYCLS365
Human PGGTaseI  300STQDRLVGGFAKWPDSHPDALHAYFGIC327
Rat PGGTaseI    300STQDRLVGGFAKWPDSHPDALHAYFGIC327
Yeast PGGTaseI  320RTQKTLTGGFSKNDEEDADLYHSCLGSA347
Pombe PGGTaseI  392HAQHAL-GGFSKTPGEFPDVLHSALGLY318
Yeast PGGTaseII 266C-QDEKKGGISDRPENEVDVFHTVFGVA292
Rat PGGTaseII   270C-QDEETGGFADRPGDMVDPFHTLFGIA296
                    *     *. .       *  *.
```

FIGURE 1: Alignment of amino acid sequences for the β-subunits of protein prenyltransferases. The alignments included PFTase from humans (Omer et al., 1993), cows (Omer et al., 1993), rats (Chen et al., 1991), pea (Yang et al., 1993) and *S. cerevisiae* (Goodman et al., 1988); PGGTase-I from rats (Zheng et al., 1994), humans (Zheng et al., 1994), *Schizosaccharomyces pombe cwg*$^{2+}$ (Diaz et al., 1993), and *S. cerevisiae* (Ohya et al., 1991); PGGTase-II from rats (Armstrong et al., 1993) and *S. cerevisiae* (Rossi et al., 1991) using the CLUSTAL program within PC Gene. Asterisks indicate amino acids which are identical and dots indicate those which are similar. Amino acids mutated in this study are shown in boldface letters.

Table 1: Site-Directed Mutations in Yeast PFTase and Diagnostic Restriction Sites

plasmid	restriction site	site-directed mutations: nucleic acid(s)	site-directed mutations: amino acid	mutant name
pGP14				
pJMD156	*Nhe* I	CAT to GCT	His156 to Ala	H156A
pJMD193	*Eco*RV	AAA to GCA	Lys193 to Ala	K193A
pJMD196	*Eco*RV	AAT to GCT	Asn196 to Ala	N196A
pJMD209	*Apa*LI	GAT to GCA	Asp209 to Ala	D209A
pJMD211	*Apa*LI	AGA to CCA	Arg211 to Gln	R211Q
pJMD256	*Afl*III	GAG to GCT	Glu256 to Ala	E256A
pJMD301	*Sal*I	AGG to CAA	Arg301 to Gln	R301Q
pJMD304	*Sal*I	AAA to GCC	Lys304 to Ala	K304A
pJMD307	*Sal*I	GAC to GCC	Asp307 to Ala	D307A
pJMD309	*Sal*I	TGC to GCC	Cys309 to Ala	C309A
pJMD310	*Sal*I	TAT to TTC	Tyr310 to Phe	Y310F
pJMD344		CAA to GCA	Gln344 to Ala	Q344A
pJMD363	*Nar*I	CAT to GCT	His363 to Ala	H363A

and eluted with 5 mM Asp-Phe. A final chromatography on a Poros HQ column provided enzymes that were 90−95% pure by SDS−PAGE.

All of the enzymes gave two bands upon SDS−PAGE characteristic of the Ram1 and Ram2 subunits. Since the Glu-Glu-Phe affinity tag was only present on the Ram1 protein, none of the mutations appeared to significantly alter subunit association. However, this observation did not rule out more subtle conformational changes. Wild-type PFTase and all of the mutants were also examined by circular dichroism spectroscopy from 190−250 nm.

The wild-type enzyme and all of the mutant enzymes had similar profiles, indicating that no major conformation changes occurred due to the single amino acid mutations. Removal of Zn^{2+} by dialysis with *o*-phenanthroline did not change the CD profiles.

Kinetic Studies. The steady-state kinetic properties of wt and mutant PFTases were analyzed using a continuous fluorescence assay (Pompliano et al., 1992b) as modified for yeast PFTase (Cassidy et al., 1995). This assay measures the increase in fluorescence upon farnesylation of a dansylated peptide, dansyl-GCVIA (Pep). Michaelis constants (K_M) for Pep and FPP and catalytic constants (k_{cat}) were determined for all enzymes. The results are summarized in Table 2. Initial velocity versus substrate concentrations were fit to the standard Michaelis−Menten equation using a nonlinear regression analysis. Substrate inhibition was observed with dansyl-GCVIA at concentrations above its K_M^{Pep} value (Dolence et al., 1995). Therefore, when FPP was the varied substrate, the peptide concentration was held

Table 2: Kinetic Constants for Yeast PFTase and Site-Directed Mutants

enzyme	k_{cat} (s^{-1})	k_{cat}^{Rel}	K_M^{PEP} (μM)	$K_M^{FPP(App)}$ (μM)
wt	4.5 ± 1.9	1	0.9 ± 0.1	0.82 ± 0.1
H156A	2.2 ± 0.14	0.49	3.9 ± 0.6	0.43 ± 0.04
K193A	8.7 ± 3.5	1.9	3.7 ± 0.5	1.2 ± 0.2
N196A	4.8 ± 0.8	1	3.2 ± 1.1	0.93 ± 0.1
D209A	0.72 ± 0.02	0.16	13.7 ± 1.3	0.12 ± 0.03
R211Q	0.0079 ± 0.0004	0.0018	1.6 ± 0.2	0.46 ± 0.05
E256A	0.058 ± 0.002	0.013	1.8 ± 0.14	106 ± 12
R301Q	0.21 ± 0.01	0.047	0.9 ± 0.1	1.5 ± 0.2
K304A	0.60 ± 0.04	0.13	0.9 ± 0.2	0.87 ± 0.1
D307A	0.012 ± 0.001	0.0027	1.1 ± 0.14	0.12 ± 0.01
C309A	0.01 ± 0.0004	0.0022	0.45 ± 0.05	0.35 ± 0.02
Y310F	0.032 ± 0.001	0.0071	0.094 ± 0.01	0.029 ± 0.004
Q344A	0.62 ± 0.04	0.14	1.7 ± 0.3	3.8 ± 0.6
H363A	0.0066 ± 0.0004	0.0015	1.3 ± 0.2	0.32 ± 0.03

where maximal activity was obtained for wt PFTase (2.4 μM) for all mutants except D209A, and the K_Ms obtained are apparent values. For D209A, the peptide concentration was held at 15 μM. Problems with solubility in the assay buffer precluded using higher peptide concentrations.

Six of the mutants, H156A, K193A, N196A, D209A, K304A, and Q344A, were good catalysts, with k_{cat}s and K_Ms within a factor of 15 of the corresponding values for wt enzyme. The remaining seven enzymes were substantially poorer catalysts, and five of these, R211Q, D307A, C309A, Y310F, and H363A, had much lower k_{cat}s, ranging from 1.5×10^{-3} to 7.1×10^{-3} of the wt values. Two of the mutants had significantly larger Michaelis constants. K_M^{Pep} for D209A was 15-fold higher than wt, while K_M^{FPP} for the E256A enzyme was elevated 130-fold. In both cases, K_M for the other substrate was similar to the wt value. For the Y310F mutant, K_Ms for both substrates were substantially lower.

Zn^{2+} Binding Studies. wt PFTase and the R211Q, D307A, C309A, Y310F, and H363A mutants were dialyzed against metal-free buffer and then analyzed for zinc content. The averages of two analyses are shown in Table 3. wt enzyme and the R211Q and Y310F mutants retained one atom of zinc per heterodimer. Measurement of the activity of wt PFTase also indicated that the enzyme retains its catalytic Zn atom. In contrast, the D307A, C309A, and H363A mutants retained less than 0.5 atom of zinc. Interestingly, EDTA did not remove Zn from yeast PFTase but was able to generate the apo mammalian enzyme (Reiss et al., 1992).

Zn^{2+} Depletion and Reconstitution Studies. During experiments to obtain apoenzymes by incubation of the purified proteins in buffer containing EDTA and 1,10-phenanthroline, we discovered that the H156A mutant lost zinc somewhat faster than wt PFTase. The pseudo-first-order rate constants for removal of Zn^{2+} by 25 mM EDTA and 2 mM 1,10-phenanthroline were $k^{wt} = 0.15 \pm 0.01\ s^{-1}$ and $k^{H156A} = 0.053 \pm 0.005\ s^{-1}$, respectively. In addition, the H156A mutant recovered activity more slowly when incubated in buffer containing 10 μM Zn^{2+}. After 8 h, only 30% of the original activity of the H156A mutant was recovered, whereas wt PFTase had recovered a similar level of activity after only 5 min. Limitations related to the sensitivity of the fluorescence assay precluded similar experiments with the less active D307A, C309A, and H363A mutants.

DISCUSSION

PFTase, PGGTase-I, and PGGTase-II constitute a superfamily of protein prenyltransferases in eukaryotes that catalyze the posttranslational alkylation of cysteine residues. The enzymes are heterodimers. Each has a distinctive β-subunit. The α-subunit in PGGTase-II is also distinctive, but PFTase and PGGTase-I have identical α-subunits. Clearly, the β-subunits in PFTase and PGGTase-I confer unique binding properties on the two enzymes which permit them to recognize different protein and isoprenoid substrates.

Multiple amino acid sequence alignments for the β-subunits of PFTase, PGGTase-I, and PGGTase-II from a variety of eukaryotes revealed five regions of similarity. Within these regions were several polar and charged residues that are likely candidates for substrate and metal binding. Based on structural studies of Zn^{2+} binding sites in other proteins, the conserved cysteine, histidine, aspartate, and glutamate residues are all candidates for the Zn^{2+} sites (Vallee & Auld, 1993). Although Mg^{2+} is a common cofactor for prenyltransferases, the active site architecture of the Mg^{2+} site is known for only a single enzyme, farnesyl diphosphate synthase. An X-ray structure of the enzyme containing Mg^{2+} and a diphosphate substrate shows the metal ion is surrounded by oxygens from the diphosphate moiety in the substrate and the carboxylates in a highly conserved DDxxD motif (Tarshis et al., 1996). The enzyme−substrate−Mg^{2+} complex is also stabilized by hydrogen bonds to arginine, lysine, and glutamine residues. Thus, the polar and charged amino acids in the 5 conserved regions of the β-subunits in protein prenyltransferses are likely candidates for site-directed mutagenesis to assess their potential roles in binding and catalysis, and we selected 13 of these residues for study.

Recombinant wt and mutant enzymes were purified by immunoaffinity chromatography on an anti-α-tubulin column. The codons used to append the C-terminal -EEF recognition motif to the β-subunits of the enzymes were also arranged to facilitate translational coupling of the open reading frames for the α- and β-subunits during expression in order to enhance the level of protein synthesis (Mayer et al., 1993). All of the enzymes eluted as heterodimers. Thus, neither the -EEF appendage nor the mutations disrupted association between the subunits. CD spectra of the wild-type and mutant enzymes were similar, indicating that the amino acid point substitutions did not grossly alter the conformation of the heterodimers. In addition, the overall shapes of the spectra are consistent with a high degree of helical character in the enzymes (Garner et al., 1978).

All of the PFTase mutants catalyzed farnesylation of the peptide substrate. The steady-state kinetic constants k_{cat} and K_M for the H156A, K193A, N196A, K304A, and Q344A mutants were within a factor of 7 of those for the wild-type enzyme, with the largest deviations in k_{cat}. The catalytic constant for the R301Q mutant was 20-fold lower than for wt enzyme, although the K_M's for both Pep and FPP were

Table 3: Zinc Analysis of Wild-Type PFTase and Mutants

enzyme	wild-type	R211Q	D307A	C309A	Y310F	H363A
mol of Zn/mol of enzyme	1.0	1.1 ± 0.3	0.3 ± 0.1	0.4 ± 0.1	1.6 ± 0.5	0.3 ± 0.2

substantially unaltered. The D209A mutant had a K_M^{Pep} that was 15-fold higher than the corresponding value for the wt PFTase, suggesting that the residue is involved in binding the peptide substrate. The E256A mutant had a somewhat lower k_{cat} (20-fold) and a substantially higher $K_M^{FPP(App)}$ (130-fold). The change in K_M is consistent with a role for Glu^{256} in FPP binding. The remaining mutants, R211Q, D307A, C309A, Y310F, and H363A, all had k_{cat}s at least 100-fold smaller than the wt enzyme, although the Michaelis constants for both substrates were all within a factor of 10 of the wt values. The results suggest a role for these residues that directly influences catalysis.

In a recent publication, Fu et al. (1996) used a differential labeling technique to search for cysteine residues in the catalytic site of rat PFTase. They discovered a residue, Cys^{299} in the β-subunit, that was only labeled by *N*-ethylmaleimide after the essential Zn^{2+} had been removed. Replacement of Cys^{299} by alanine or serine gave mutant enzymes that had lost essentially all catalytic activity as well as their ability to bind Zn^{2+}, although they retained their FPP binding properties. Fu et al. proposed that Cys^{299} is likely to be one of the residues that directly coordinate the zinc atom. Cys^{299} in rat PFTase corresponds to Cys^{309} in the yeast enzyme. Our C309A mutant was also catalytically compromised and, like its rat counterpart, was compromised in its ability to bind Zn^{2+}. The D307A and H363A mutants had similar kinetic and Zn^{2+} binding properties. These similarities among the D307A, C309A, and H363A mutants suggest similar roles for the amino acids. While there are several logical explanations for these results, the most economical is that the effects of Asp^{307}, Cys^{309}, and His^{363} are primarily through Zn^{2+} binding, and the reduced catalytic efficiency is due primarily to loss or misalignment of the metal. The residual activity for these mutants could represent the basal prenyltransferase activity for the apoenzyme. This activity could reflect a normal level of activity for a small equilibrium population mutant with an atom of Zn^{2+} in the altered metal binding site, or it could reflect a misalignment of Zn^{2+} in the catalytic site.

In contrast, although the R211Q and Y310F mutants were poor catalysts, they retained their Zn^{2+} binding properties. The roles of Arg^{211} and Tyr^{310} are less clear. Arg^{211} might assist in activating the diphosphate moiety by a direct interaction with nonbridging oxygen atoms in the pyrophosphate leaving group. A similar role was proposed for an arginine in farnesyl diphosphate synthase, where the guanidinium group is directly bound to the diphosphate moiety of the allylic isoprenoid substrate (Tarshis et al., 1996).

Although the catalytic constants of the H156A enzyme are similar to those of wt PFTase, the mutant lost Zn^{2+} 3 times faster upon treatment with chelating reagents. Also, the apo H156A mutant regained activity approximately 100 times more slowly than apo wt enzyme when incubated in buffer containing 10 μM Zn^{2+}. In comparison with the Asp^{307}, Cys^{309}, and His^{363} mutants, H156A does not appear to have a direct role in Zn^{2+} binding but may help maintain the conformational integrity of the enzyme in a manner that alters the rates of release and uptake of the metal ion.

In conclusion, site-directed mutagenesis of conserved polar and charged amino acids in the β-subunit of yeast PFTase was used to identify five residues that were important for catalysis. Three of these amino acids, Asp^{307}, Cys^{309}, and His^{363}, also were essential for optimal binding of Zn^{2+}. The conserved nature of these residues suggests that they play similar roles in PGGTase-I and PGGTase-II as well.

ADDED IN PROOF

Subsequent to submission of this paper, the crystal structure of rat PFTase was published (Park et al., 1997). The putative active site is composed of two clefts that intersect at a bound zinc atom. The authors identified three residues which coordinate the zinc ion, $Asp^{297\beta}$, $Cys^{299\beta}$, and $His^{362\beta}$. These residues correspond to Asp^{307}, Cys^{309}, and H^{363} in yeast PFTase which we found are deficient in their ability to bind the zinc ion during dialysis.

REFERENCES

Andres, D. A., Goldstein, J. L., Ho, Y. K., & Brown, M. S. (1993) *J. Biol. Chem. 268*, 1383−1390.

Armstrong, S. A., Seabra, M. C., Sudhof, T. C., Goldstein, J. L., & Brown, M. S. (1993) *J. Biol. Chem. 268*, 12221−12229.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Caplin, B. E., Hettich, L. A., & Marshall, M. S. (1994) *Biochim. Biophys. Acta 1205*, 39−48.

Cassidy, P. B., Dolence, J. M., & Poulter, C. D. (1995) *Methods Enzymol. 250*, 30−43.

Chen, W.-J., Andres, D. A., Goldstein, J. L., Russell, D. W., & Brown M. S. (1991) *Cell 66*, 327−334.

Chen, W.-J., Moomaw, J. F., Overton, L., Kost, T. A., & Casey, P. J. (1993) *J. Biol. Chem. 268*, 9675−9680.

Clarke, S. (1992) *Annu. Rev. Biochem. 61*, 355−386.

Davisson, V. J., Woodside, A. B., Neal, T. R., Stremler, K. E., Muehlbacher, M. & Poulter, C. D. (1986) *J. Org. Chem. 51*, 4768−4779.

Diaz, M., Sanchez, Y., Bennett, T., Sun, C. R., Godoy, C., Tamanoi, F., Duran, A., & Perez, P. (1993) *EMBO J. 12*, 5245−5254.

Dolence, J. M., Cassidy, P. B., Mathis, J. R., & Poulter, C. D. (1995) *Biochemistry 34*, 16687−16694.

Fu, H. W., Moomaw, J. F., Moomaw C. R., & Casey, P. J. (1996) *J. Biol. Chem. 271*, 28541−28548.

Furfine, E. S., Leban, J. J., Landavazo, A., Moomaw, J. F., & Casey, P. J. (1995) *Biochemistry 34*, 6857−6862.

Garner, J., Osguthorpe, D. J., & Robson, B. (1978) *J. Mol. Biol. 120*, 97−120.

Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319−326.

Goodman, L. E., Perou, C. M., Fujiyama, A., & Tamanoi, F. (1988) *Yeast 4*, 271−281.

Higgins, D. G., & Sharp, P. M. (1988) *Gene 73*, 237−244.

Horiuchi, H., Kawata, M., Katayama, M., Yoshida, Y., Musha, T., Ando, S., & Takai, Y. (1991) *J. Biol. Chem. 266*, 16981−16984.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.

Mathis, J. R., & Poulter, C. D. (1997) *Biochemistry 36*, 6367−6376.

Mayer, M. P., Prestwich, G. D., Dolence, J. M., Bond, P. D., Wu, H.-Y., & Poulter, C. D. (1993) *Gene 132*, 41−47.

Mitsuzawa, H., Esson, K., & Tamanoi, F. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1704−1708.

Moomaw, J. F., & Casey, P. J. (1992) *J. Biol. Chem. 267*, 17438−17443.

Moores, S. L., Schaber, M. D., Mosser, S. D., Rands, E., O'Hara, M. B., Garsky, V. M., Marshall, M. S., Pompliano, D. L., & Gibbs, J. B. (1991) *J. Biol. Chem. 266*, 14603−14610.

Ohya, Y., Goebl, M., Goodman, L. E., Petersen-Bjorn, S., Friesen, J. D., Tamanoi, F., & Anraku, Y. (1991) *J. Biol. Chem. 266*, 12356−12360.

Omer, C. A., Krai, A. M., Diehl, R. E., Prendergast, G. C., Powers, S., Allen, C. M., Gibbs, J. B., & Kohl, N. E. (1993) *Biochemistry 32*, 5167−5176.

Park, H.-W., Boduluri, S. R., Moomaw, J. F., Casey, P. J., & Beese, L. S. (1997) *Science 275*, 1800−1804.

Pompliano, D. L., Rands, E., Schaber, M. D., Mosser, S. D., Anthony, N. J., & Gibbs, J. B. (1992a) *Biochemistry 31*, 3800−3807.

Pompliano, D. L., Gomez, R. P., & Anthony, N. J. (1992b) *J. Am. Chem. Soc. 114*, 7945−7946.

Pompliano, D. L., Schaber, M. D., Mosser, S. D., Omer, C. A., Shafer, J. A., & Gibbs, J. B. (1993) *Biochemistry 32*, 8341−8347.

Reiss, Y., Goldstein, J. L., Seabra, M. C., Casey, P. J., & Brown, M. S. (1990a) *Cell 62*, 81−88.

Reiss, Y., Seabra, M. C., Goldstein, J. L., & Brown, M. S. (1990b) *Methods 1*, 241−245.

Reiss, Y., Stradley, S. J., Gierasch, L. M., Brown, M. S., & Goldstein, J. L. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 732−736.

Reiss, Y., Brown, M. S., & Goldstein, J. L. (1992) *J. Biol. Chem. 267*, 6403−6408.

Rossi, G., Jiang, Y., Newman, A. P., & Ferro-Novick, S. (1991) *Nature 351*, 158−161.

Sagami, H., Ogura, K., Weiner, A., & Poulter, C. D. (1984) *Biochem. Int. 8*, 661−667.

Seabra, M. C., Goldstein, J. L., Sudhof, T. C., & Brown, M. S. (1992) *J. Biol. Chem. 267*, 14497−14503.

Tarshis, L. C., Proteau, P. J., Kellogg, B. A., Sacchettini, J. C., & Poulter, C. D. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 15018−15023.

Vallee, B. L., & Auld, D. S. (1993) *Acc. Chem. Res. 26*, 543−551.

Witter, D. J., & Poulter, C. D. (1996) *Biochemistry 35*, 10454−10463.

Yang, Z., Cramer, C. L., & Watson, J. C. (1993) *Plant Physiol. 101*, 667−674.

Yokoyama, K., Goodwin, G. W., Ghomashchi, F., Glomset, J. A., & Gelb, M. H. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5302−5306.

Yokoyama, K., McGeady, P., & Gelb, M. H. (1995) *Biochemistry 34*, 1344−1354.

Zhang, F. L., Diehl, R. E., Kohl, N. E., Gibbs, J. B., Giros, B., Casey, P. J., & Omer, C. A. (1994) *J. Biol. Chem. 269*, 3175−3180.

BI970039P