In Vitro Phosphorylation By cAMP-dependent Protein Kinase Upregulates Recombinant
S. cerevisiae Mannosylphospho Dolichol Synthase

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Abbreviations

DPMS, Dol-P-Man synthase; PKA, cAMP-dependent protein kinase; DTT, Dithiothreitol; EDTA, Ethylenediaminetetra acetic acid; BSA, Bovine serum albumin; Me$_2$SO, Dimethyl sulfoxide; PMSF, Phenylmethylsulfonyl fluoride; TPCK, 1-Tosylamide 2-phenylethyl chloromethyl ketone; SDS, Sodium dodecyl sulfate; PAGE, Polyacrylamide gel electrophoresis; IPTG, Isopropyl β-D-thiogalacto pyranoside; HRP, Horseradish peroxidase.
Abstract

Dpm1 is the structural gene for mannosylphospho dolichol synthase (i.e., Dol-P-Man synthase, DPMS) in *S. cerevisiae*. Earlier studies with cDNA cloning and sequence analysis have established that M, 31 kDa DPMS of *S. cerevisiae* contains a consensus sequence (YRRVIS) that can be phosphorylated by cAMP-dependent protein kinase (PKA). We have been studying the upregulation of DPMS activity by PKA-mediated phosphorylation in higher eukaryotes, and used the recombinant DPMS from *S. cerevisiae* in this study to advance our knowledge further. DPMS catalytic activity was indeed enhanced several folds when the recombinant protein was phosphorylated *in vitro*. The rate as well as the magnitude of catalysis was higher with the phosphorylated enzyme. A similar increase in the catalytic activity was also observed when the *in vitro* phosphorylated recombinant DPMS was assayed as a function of increasing concentrations of exogenous dolichylmonophosphate (Dol-P). Kinetic studies indicated that there was no change in the K for GDP-mannose between the *in vitro* phosphorylated and control recombinant DPMS, but the V was increased by 6-fold with the phosphorylated enzyme. *In vitro* phosphorylated recombinant DPMS also exhibited higher enzyme turnover (kcat) and the enzyme efficiency (kcat/Km). SDS-PAGE followed by autoradiography of the 32P-labeled DPMS detected a M, 31-kDa phosphoprotein, and immunoblotting with anti-phosphoserine antibody established the presence of a phosphoserine residue in *in vitro* phosphorylated recombinant DPMS. To confirm the phosphorylation activation of recombinant DPMS, serine-141 in the consensus sequence was replaced with alanine by PCR-site directed mutagenesis. S141A DPMS mutant exhibited 50% reduction in catalytic activity compared to the wild type when both were analyzed after *in vitro* phosphorylation. Thus, confirming that *S. cerevisiae* DPMS activity is indeed regulated by the cAMP-dependent protein phosphorylation.
signal, and the phosphorylation target is serine-141.

**Introduction**

Mannnosylphospho dolichol (Dol-P-Man), a mannosyl donor in the assembly of the precursor oligosaccharide-lipid Glc₃Man₉GlcNAc₂-PP-Dol in N-glycosylation of proteins, in the synthesis of glycosylphosphatidylinositol (GPI) anchors, in O-glycosylation of proteins in yeast, and in C-mannosylation of Trp-7 in human ribonucleasae 2 (RNase 2) is formed by the transfer of mannose from GDP-Mannose to the polyisoprenoid-lipid, dolichyl monophosphate (Dol-P; 1-7). Dol-P-Man is synthesized at the cytoplasmic face of the endoplasmic reticulum (ER) membrane (8-10), and catalyzed by mannosylphospho dolichol synthase (DPMS; Dol-P-Man synthase, EC 2.4.1.83). Dol-P-Man synthase deficiency has been observed in Class E Thy-1 lymphoma patient and is unable to elongate Man₅GlcNAc₂-PP-Dol to Man₉GlcNAc₂-PP-Dol, a pre-requisite for Glc₃Man₉GlcNAc₂-PP-Dol synthesis (11). We have also made a similar observation using in vitro studies with amphomycin, a lipopeptide antibiotic from *S. canas*, which forms a complex with Dol-P in a calcium dependent manner, and inhibiting the Dol-P-Man synthase activity (12,13). Recent reports indicate that partial deficiency of Dol-P-Man synthase causes congenital disorder of glycosylation (CDG; 14,15). DPMS deficiency in these patients is associated with developmental delay, seizures, hypotonia and dysmorphic function (16). The DPM1 gene is essential for viability in yeast since disruption of the gene is lethal (17).

Dol-P-Man synthase has been partially purified from mammalian tissue (18,19) and highly purified from the budding yeast, *S. cerevisiae* (20). Cloning of Dol-P-Man synthase gene Dpm1p from *S. cerevisiae*, has shown DPMS to be a structural gene in yeast (17) expressing active protein both in *Escherichia coli* and in mammalian cells (21). Information on the primary structure of Dol-P-Man synthase obtained from cDNA analysis has indicated that (i) it codes for a protein of 267 amino acids with an apparent mass of 30.36-kDa; (ii) there is a potential
membrane spanning domain of 25 amino acids at its carboxy terminus; (iii) a highly conserved amino acid sequence in the membrane spanning domain originally suggested as a potential dolichol and/or polyisoprene substrate recognition site; and (iv) the predicted sequence contains one positive site for phosphorylation by cAMP-dependent protein kinase (i.e., serine-141 in a sequence RRVIS\textsuperscript{141}, a consensus sequence for phosphorylation; 17, 22,23). Expression of yeast Dol-P-Man synthase in \textit{E. coli} and characterization of the purified recombinant enzyme revealed it as a 31-kDa protein. The amino acid composition and sequence of the conserved domain are not critically important for the recognition and binding of Dol-P when the synthase is present in a lipid matrix (24). However, the Dol-P-Man synthase from \textit{S. cerevisiae} is different from the mammalian Dol-P-Man synthase in its \textit{in vitro} properties such as sensitivity to nonionic detergents and its ability to interact with phospholipid vesicles (24-26).

Dol-P-Man synthase has now been cloned from a number of species such as \textit{Trypanosoma brucei}, \textit{Ustilago Maydis}, \textit{Schizosaccharomyces pombe}, \textit{Caenorhabditis briggsiae} as well as from humans by complementation of a temperature-sensitive \textit{S. cerevisiae} Dpm1 mutant, or by cDNA cloning (27-29). Sequence analysis has suggested that the Dol-P-Man synthase can now be divided into two classes: One includes the enzymes from \textit{S. cerevisiae}, \textit{Ustilago maydis}, \textit{Trypanosoma brucei}, and \textit{Leishmania mexicana} (29,30). They share 50-60% amino acid identity and have a stretch of hydrophobic amino acid residues near the C-terminus constituting a transmembrane domain. The other includes enzymes from the human, \textit{S. pombe}, and \textit{Caenorhabditis briggsiae}, lack the hydrophobic C-terminus domain, and, thus, the transmembrane domain. In addition, they have also exhibited only 30% amino acid identity with the other group (21,25,30). Yeast Dpm1 DNA complemented both mouse \textit{Thy-1} negative lymphoma mutant cells of complementation class E and the Lec15 mutant of Chinese hamster cells (31,32). On the other hand, human and mouse homologs of Dpm1, hDpm1 and mDpm1 did not complement the DPMS mutant in Lec15 cells. This led to establish that mammalian DPMS is a multi-component enzyme represented by the catalytic subunit Dpm1 and two accessory
proteins DPM2 and DPM3 (33-35). Most striking however, is that Dol-P-Man synthase from every known source has a serine residue in the position corresponding to serine-141 in S. cerevisiae DPMS. Serine-141, and the preceding conserved residues meet the criteria for a consensus site for phosphorylation by cAMP-dependent protein kinase in the DPMS (22,23). In addition, a cDNA encoding the Schistosoma mansoni DPMS displaying a high homology with Cricetulus griseus and S. pombe (36) as well as a probable DPMS sequence (241 amino acid residues) in the genome sequence of the fruit fly, Drosophila melanogaster (37) have also been identified.

We have been studying the regulation of Dol-P-Man synthase activity in mammalian cells by a cAMP-dependent protein phosphorylation signal (38). Our results supported enhanced Dol-P-Man synthase activity in ER membranes from cells treated with a β-agonist isoproterenol, as well as after in vitro phosphorylation of the ER membranes by the catalytic subunit of the cAMP-dependent protein kinase (39,40). The increased enzyme activity was due to an increase in the $V_{\text{max}}$ and was independent of enhanced transcription (41). This was strongly supported by the down regulation of the synthase activity in a series of cAMP-dependent protein kinase (PKA)-deficient Chinese hamster ovary (CHO) cell mutants (42). To understand the molecular detail of the phosphorylation regulation of Dol-P-Man synthase, we have used here a purified recombinant Dol-P-Man synthase from S. cerevisiae (wild type) as well as a DPMS mutant in which serine-141 has been replaced with alanine (S141A mutant). We report here that both biochemical and kinetic data have supported upregulation of the wild type recombinant Dol-P-Man synthase activity after in vitro phosphorylation by PKA. SDS-PAGE followed by autoradiography of the $[^{32}\text{P}]$-labeled Dol-P-Man synthase has detected a $M_r$ 31-kDa phosphoprotein species as well. Identification of the 31-kDa phosphoprotein on the immunoblot
probed with anti-phosphoserine antibody further supported that Dol-P-Man synthase is phosphorylated at the Ser-141. To evaluate serine-141 as the phosphorylation target, we have developed a phosphorylation site deficient DPMS mutant in which serine-141 has been replaced with alanine by PCR site-directed mutagenesis. The S141A DPMS mutant has been successfully expressed as a functionally active enzyme in E. coli and analyzed after in vitro phosphorylation by PKA.

**Materials and Methods**

*E. coli* DH5α harboring plasmid pDPM6 containing the structural gene Dpm1 for Dol-P-Man synthase was originally developed in Dr. P.W. Robbins laboratory at MIT and obtained from Dr. J.S. Schutzbach. Dolihyl monophosphate, ATP (Na salt), catalytic subunit of cAMP-dependent protein kinase (PKA, bovine heart), bovine serum albumin (crystalline), Chloramphenicol, Ampicillin, IPTG, PMSF, TPCK, soybean trypsin inhibitor, leupeptin, aprotinin, Protein A-Sepharose CL-4B and biotin-conjugated anti-phosphoserine monoclonal antibody were purchased from Sigma Chemical Co., St. Louis, MO. Protein molecular weight markers (high molecular weight, low molecular weight, and kaleidoscope), reagents for protein electrophoresis and immunoblotting, and hydroxyapatite were from Bio-Rad Laboratories, Hercules, CA. GDP- [U-14C] Mannose (307 mCi/mmol; a Ci = 37 Gbq), gamma- [32P] ATP (3.0 Ci/ mmol), [14C] methylated protein mixture, ECL chemiluminescence kit and Amplify were obtained from Amersham Pharmacia Biotech, Newark, NJ. Mouse monoclonal antibody to yeast Dol-P-Man synthase was obtained from Molecular Probes, Eugene, OR. Oligonucleotides for PCR primers were obtained from Oligos Slc, Ridgefield, CT as well as from the Molecular Biology Core Facility of the University of Puerto Rico Medical Sciences Campus. Restriction enzymes were acquired from New England BioLab, Boston, MA.
Analytical Methods: Protein was assayed following precipitation of the protein with trichloroacetic acid using a modification (42) of the procedure of Lowry et al (43). Bovine serum albumin was used as the standard. Electrophoresis was carried out under reducing conditions in 12% polyacrylamide gels (SDS-PAGE) using the buffer system described by Laemmli (44). The gels were either processed for autoradiography in Amplify™, according to the procedure described by Bonner and Lasky (45), or they were processed for Western Blot analysis.

Buffers: Buffer A: 25 mM sodium phosphate, pH 8.0 containing 5 mM MgCl₂ and 0.2% β-mercaptoethanol. Buffer B: 10 mM sodium phosphate, pH 8.0 containing 0.5 mM EDTA, 10% glycerol, 0.1% Nonidet P-40 and 1.0 mM dithiothreitol. Buffer C: 100 mM Tris-acetate, pH 7.5 containing 10% glycerol (v/v), 0.2% β-mercaptoethanol and 0.1% sodium dodecyl sulfate (w/v): NET Buffer: 50 mM Tris-HCl, pH 7.4 containing 150 mM NaCl, 5 mM EDTA, 0.02% NaN₃ and 2% BSA; Washing Buffer: 100 mM Tris-HCl, pH 7.4, 1% β-mercaptoethanol, and 50 mM LiCl; TTBS: 20 mM Tris-HCl, pH 7.5 containing 137 mM NaCl and 0.05% Tween 20.

Dol-P-Man Synthase Assay: Enzymatic transfer of mannose from GDP-Mannose was assayed using the procedure described earlier (13) with the following modifications. In vitro phosphorylated or control recombinant Dol-P-Man synthase was incubated at 37°C for 2 min in 30 mM Hepes, pH 7.0 buffer containing 150 µM EDTA, 30 µM DTT, 3% glycerol, 90 mM NaCl, 0.25% Triton X-100, 10 mM MnCl₂, 10 µg Dol-P, 1% Me₂SO and 0.125 µM GDP-[¹⁴C]mannose (Sp. Act. 318 cpm/pmol) in a total volume of 100 µl, unless otherwise mentioned. The reaction was stopped with 2 ml of chloroform-methanol (2:1, v/v). Chloroform-methanol extracts containing the Dol-P-Man were washed once with 5 volumes of 0.9% sodium chloride and twice with chloroform-methanol-water (3:47:48, v/v/v) to remove free GDP-[¹⁴C]-mannose. Lower organic phase containing the Dol-P-Man was then quantified in a liquid scintillation...
Recombinant Dol-P-Man synthase was phosphorylated in vitro by incubating with the catalytic subunit from cAMP-dependent protein kinase (12 units per 30 µg protein) at 30°C for 20 min in the presence of 10 mM NaF, 10 mM MgCl₂ and 200 µM ATP as described earlier (42). In some experiments, the reactions were started by the addition of 5 µCi of gamma-[³²P]ATP (Sp. Act. 3.0 µCi/mmol).

**Construction of the Expression vector BL21(DE3)pDPM1-9 and Preparation of the Enzyme:**

The expression vector was constructed earlier by Schutzbach et al to purify the Dol-P-Man synthase (46). In brief, M9ZB Broth (47; 4x250 ml in 1 liter flasks) containing 100 µg/ml ampicillin was inoculated with 10 ml of an overnight culture of BL21(DE3)/pDPM1-9. Cultures were incubated at 37°C on a shaker at 200 rpm. After 3 h (A₆₀₀ nm of 0.66) the enzyme was induced by adding 2.5 ml of 100 mM IPTG. The cells were harvested 2 h post-induction, by centrifugation at 6,000 rpm for 15 min (Sorvall GSA rotor). All additional procedures were carried out at 0-4°C or on ice. The cell pellets were washed with 300 ml of water followed by 100 ml of Buffer A, and frozen overnight. The pellets were then thawed, suspended in 10 ml of Buffer A, and the cells were sonically ruptured three times for 30 sec with 1-min intervals to allow cooling using a Branson sonifier (Kontes, model W185) at a power setting of 4.5. The suspension was fractionated by centrifugation at 39,000 xg for 20 min (Sorvall SS-34 rotor) at 4°C. The pellet was washed twice with 25 ml of Buffer A and then suspended in 5 ml of the same buffer. The suspension was diluted with 17.5 ml of Buffer B and 2.5 ml of 10% Nonidet P-40 was added. The mixture was centrifuged at 18,000 rpm for 20 min. Both supernatant (which often contained significant amounts of activity), and the pellet were saved. To solubilize additional enzyme, the pellet was suspended in 20 ml of Buffer C. To the dispersed pellets 2.5 ml of 10% Nonidet P-40 was added and homogenized for 10 min in a Tekmer homogenizer. The homogenates were centrifuged as above, and the supernatants were saved. The procedure...
was repeated once more to yield a third solubilized fraction. Solubilized fractions containing the enzyme activity were pooled and applied to a hydroxyapatite column (2.5 x 11 cm) equilibrated with Buffer B. The column was washed with 30 ml of Buffer B and eluted with a 400 ml linear gradient of 0.0 to 1.0 M NaCl in the same buffer. Fractions containing the enzyme activity were pooled and concentrated by ultrafiltration using YM-10 membranes (Amicon).

**Immunoprecipitation and Immunoblotting:** Recombinant Dol-P-Man synthase after purification was phosphorylated *in vitro* in the presence of gamma-[^32P] ATP as described above.[^32P]-labeled Dol-P-Man synthase was immunoprecipitated with a mouse monoclonal antibody (1:1,000 dilution) to yeast DPMS for 3 h at 4°C followed by 3 h with 50 µg/ml of Protein A-Sepharose CL-4B (30 mg/ml) in the presence of aprotinin (1 µg/ml), PMSF (1mM), TPCK (200 µM), soybean trypsin inhibitor (1 µg/ml), and leupeptin (1µM). The Protein A-Sepharose: antigen-antibody complexes were washed twice with NET buffer, twice with washing buffer, and once with PBS, pH 7.4. The immune complexes were released by boiling at 100°C for 5 min and analyzed by SDS-PAGE (12%) followed by autoradiography (48).

For immunoblotting, the recombinant Dol-P-Man synthase before and after *in vitro* phosphorylation was separated on 12% SDS-PAGE and transblotted onto 0.2 µm nitrocellulose membranes according to the procedure described by Towbin *et al* (49). The blots were treated with a blocking solution (5% non-fat dry milk in TTBS) overnight at 15°C and subsequently with (a) anti-Dol-P-Man synthase antibody (diluted 1:2,000 in blocking solution), and (b) biotinylated anti-phosphoserine antibody (diluted 1:2,000 in blocking solution) for 3 h at room temperature. The membranes were rinsed twice with TTBS, washed with one change of TTBS for 15 min and twice more 5 min apart at room temperature. Blot (a) was treated with HRP-conjugated sheep anti-mouse IgG (1:2,000 dilution) to detect the Dol-P-Man synthase; and the blot (b) was treated with streptavidin-conjugated sheep anti-mouse IgG (1:2,000 dilution) to detect phosphoserine as well as the molecular weight markers. The blots were developed according to the instructions
provided with the ECL chemiluminescence kit and exposed to Hyperfilm™ until the desired intensity was achieved.

**Isolation of Dol-P-Man Synthase S141A Mutant by PCR Site-Directed Mutagenesis:** The strategy for PCR site-directed mutagenesis consisted on the design of oligonucleotides that contained the desired substitution, and also resulted in silent mutation with either a new restriction site or elimination of an existing one to aid the analysis. The oligonucleotides were designed with the aid of DNAsis Program (Hitachi Corp.) and using the Dpm1 (*S. cerevisiae* Dol-P-Man synthase gene sequence GenBank J04184) as template. We have used Silmut computer program (50) to identify suitable restriction site(s) for silent mutagenesis. These oligonucleotides were compatible as PCR primer pairs with the Dpm1F and Dpm1R. The primer pairs, their corresponding products and diagnostic restriction enzyme were:

Dpm1F: 5'-CGGGATCCTATGGCTAGCATCGAATACTCTGTT-3'/Dpm1R: 5'-ATTCTAGCAGTCGACGATGATGCTCTGTACA-3' (449 bp);

Dpm1F: 5'- TGTACAGACGCGTACATGCTCTGTT-3'/Dpm1R: 5'-CTGGATCCTTAAAAGACCAAATGGTATAGCTGGTAGCA-3' (409 bp). The genes were first amplified in halves and then added to a second PCR mix to fill-in the gaps by the ULTMA DNA polymerase. Hybrids served as the templates for Dpm1F and Dpm1R amplification for the complete mutant gene (824 bp). The PCR reaction conditions were 1X ULTIMA reaction buffer (10 mM Tris-HCl, pH 8.8), 10 mM KCl, 0.02% Tween 20 (v/v), 1.25 mM dNTPs, 10 mM each of the corresponding primer pair, and 1.5U ULTIMA DNA polymerase (Perkin-Elmer Corp.) in a final volume of 45 µl. The tubes were incubated at 80°C for 5 minutes in a DNA Thermal Cycler, Model 480 (Perkin-Elmer Corp.) and 5 µl of 25 mM MgCl₂ was added to a final concentration of 2.5 mM. Mineral oil was added and the mixture was incubated at 97°C for 2 minutes. Thermal cycling conditions were 95°C/1 min, 60°C/1 min, 72°C/1 min for 25 cycles. Time delay 72°C/7 min.
To generate the mutant gene, PCR products were separated on 1.5 % agarose gel in 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.5) and bands corresponding to PCR halves were excised from the gel slices. An aliquot of PCR fragment (0.3 µl) from each half was used as templates in a second PCR reaction along with Dpm1F and Dpm1R primers to generate the complete mutant gene. S141A mutant gene acquiring a Sal I restriction site was analyzed by Sal I digestion (1X Sal I buffer, 0.5 µl 100X BSA, 10U Sal I, and 5 µl of PCR mix in a total volume of 15 µl at 37°C for one hour) followed by electrophoresis (65 volts for 45 minutes) on 1.5 % agarose gel in 1X TAE. The DNA fragments (450 bp) were detected under the UV light after staining with ethidium bromide.

**Subcloning of the S141A DPMS Mutant into pET3(a)/DPM1-9 and Screening for Colonies:**

PCR products containing the DPMS mutant gene as well as the wild type gene were excised from pUC19/DPMS constructs with Avr II/Nsi I. The fragments were gel purified and ligated with T4 DNA ligase (400,000 U/ml) at 16°C overnight to pDPM1-9 (46). The ligation mixture was diluted 1:20, and 2 µl were used to transform DH5α cells (Gibco-BRL; efficiency > 1x10^8 cfu/µg DNA) following the manufacturer’s instructions. The cells were then plated on LB/Amp (50 µg/ml) plates and the colonies were directly screened for the complete gene by PCR using Dpm1F and Dpm1R primers. The wild type as well as the S141A mutant DPMS in pDPM1-9 were sequenced using the Sequenese Ver 2.0 kit (USB) according to the manufacturer’s instructions. The reaction mixtures were electrophoresed in a 5% Long Ranger/1X TBE (89 mM Tris-Base, 89 mM Boric Acid, 2.5 mM EDTA.2H₂O) gel for 1.5 hours at 50W. The gel was vacuum dried, exposed to Kodak X-OMAT X-ray films with intensifying screens (Dupont) at -70°C for three days, and developed.
**Expression and Analysis of S141A Mutant DPMS:** Transformants containing the mutated DPMS gene were grown in 10 ml LB containing ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) at 37°C in a bacterial shaker incubator with shaking at 200 rpm until the OD<sub>600nm</sub> reached between 0.6-1.0. The expression of the protein was then achieved by adding IPTG to a final concentration of 0.4 mM, and the cultures were harvested after three hours.

Aliquots from uninduced and induced cultures were pelleted at 14,000 rpm for 30 seconds. The cell pellets were boiled in 1X SDS-PAGE sample buffer for 5 minutes, and a 10 µl aliquot from each sample (representing 200 µl of cells) was loaded into 12% polyacrylamide gels and separated at 180 volts for 45 minutes according to Laemmli’s procedure (43). One gel was stained with 0.1% Coomassie Blue (R250) for protein detection, and the other gel was used for immunoblotting with yeast anti-DPMS monoclonal antibody as described above.

The cultures were scaled up, and the DPMS activity was solubilized in 1% NP-40 as mentioned above. The soluble enzyme was recovered after centrifuging at 100,000 xg for 60 min at 4°C (Beckman 50Ti rotor). DPMS content in each extract was quantified by densitometry (Bio-Rad Model GS470).

**Results**

**Effect of in vitro Phosphorylation on the Dol-P-Man Synthase Activity:** Mannosylphosphodolichol synthesis was monitored with *in vitro* phosphorylated and control recombinant DPMS from *S. cerevisiae*. The time course of the synthase activity, when examined for a period of 0 - 5 min under the conditions described above, indicated that the specific activity of Dol-P-Man synthase was several-fold higher in *in vitro* phosphorylated enzyme compared to the control (Figure 1). In addition, the Dol-P-Man synthesis remained high at all time points with the *in vitro* phosphorylated enzyme.
**Dependence of GDP-Mannose and Dol-P Concentrations on the Synthase Activity:** Based on a single point assay, the Dol-P-Man synthase activity was found to be nearly 3-fold higher (p<0.05) in *in vitro* phosphorylated enzyme compared to the control (Table I). However, the kinetic analysis at the initial rate (Figures 2a & 2b) of the recombinant DPMS indicated that the K_m for GDP-Mannose for *in vitro* phosphorylated and the control enzymes were 1.1 x 10^{-6} M and 1.2 x 10^{-6} M, respectively, whereas the corresponding V_max values were 135.8 nmol/min/mg protein and 23.6 nmol/min/mg protein, respectively. The *in vitro* phosphorylated enzyme also exhibited almost 6-fold higher turnover (k_cat = 70.9) and the enzyme efficiency (k_cat/K_m = 6.4 x 10^7) as compared to the control enzyme (k_cat = 12.1, and k_cat/K_m = 1 x 10^7) (Table II). The apparent K_m for Dol-P was approximately a magnitude higher than that of the GDP-Mannose but the values did not differ between the *in vitro* phosphorylated and the control enzyme (data not shown).

**Characterization of Dol-P-Man Synthase as a Phosphoprotein:** Recombinant Dol-P-Man synthase was phosphorylated *in vitro* in the presence of gamma-[^32P] ATP according to the conditions described under Materials and Methods. The phosphorylated enzyme was immunoprecipitated with a mouse monoclonal antibody to yeast Dol-P-Man synthase, and subjected to a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography on XAR films. It detected Dol-P-Man synthase as a Mr 31-kDa phosphoprotein (Figure 3). To identify that serine-141 is the target for PKA mediated phosphorylation, Dol-P-Man synthase was separated on a 12% SDS-PAGE after *in vitro* phosphorylation and transblotted onto a nitrocellulose membrane. One-half of the membrane was processed with anti-DPMS antibody, and the other half with anti-phosphoserine antibody. Both anti-DPMS antibody and the anti-phosphoserine antibody detected a Mr 31-kDa protein on the blots (Figure 4a & 4b) supporting that Dol-P-Man synthase was indeed phosphorylated at serine-141 by the PKA. As a control, cell extracts from IPTG-uninduced and IPTG-induced E. coli carrying the
wild type Dpm1 gene or its S141A mutant were examined by Western Blot analysis against anti-phosphoserine antibody. These results (Figure 4c) were negative suggesting the presence of no other phosphorylated protein equivalent to the 31-kDa DPMS E. coli cell extracts.

**Dol-P-Man Synthase Activity of the Phosphorylation-site deficient (S141A) Mutant After in vitro Phosphorylation by cAMP-Dependent Protein Kinase:** To establish further that serine-141 of the DPMS is indeed the target for PKA-mediated phosphorylation, serine-141 was replaced by alanine by site-directed mutagenesis. Alanine substitution at serine-141 of the Dol-P-Man synthase was confirmed by (a) restriction analysis of the DNA constructs; and (b) DNA sequencing. The restriction enzyme analysis demonstrated that the wild type Dol-P-Man synthase gene contained only BstU1 site but not Sal I site. On the other hand, the S141A Dol-P-Man synthase mutant gene contained both a BstU1 and a Sal I site. DNA sequencing of the wild type and the mutant DPMS genes demonstrated the presence of TCC sequence (a codon for serine) at the amino acid residue-141 of the wild type gene but the corresponding sequence in the mutant was GCC (a codon for alanine; data not shown). It should be noted here that there were no other sequence differences among the wild type, the S141A mutant, and the published S. cerevisiae DPMS sequence.

Analysis of the expressed protein by 12% SDS-PAGE as well as by immunoblotting with a yeast anti-DPMS monoclonal antibody indicated that IPTG-induced cultures expressed high levels of S141A mutant DPMS as a Mr 31-kDa protein (Figure 5a & 5b). DPMS activity of the mutant enzyme was determined and compared with that of the wild type before and after in vitro phosphorylation. Basal DPMS activity in S141A mutant extracts was about 1.5-fold higher than control extracts. Wild type DPMS was activated by 1.5-fold as opposed to 1-fold in S141A mutant. This means if the wild type DPMS was activated by 2-fold after phosphorylation, the S141A mutant DPMS was activated less than 2-fold (Figure 6a & 6b). E.coli cells transfected with vector alone had no DPMS activity.
Using biochemical parameters we have proposed earlier that mammalian Dol-P-Man synthase is upregulated by a cAMP-dependent protein kinase mediated phosphorylation event (39). In addition, we have also shown that the mammalian Dol-P-Man synthase activity is associated with a M, 32-kDa phosphoprotein (40). We have now shown using somatic cell genetics that Chinese hamster ovary (CHO) cells deficient in cAMP-dependent protein kinase exhibited reduced Dol-P-Man synthase activity as compared to the wild type (41). Increased enzymatic activity of \textit{in vitro} phosphorylated recombinant DPMS from \textit{S. cerevisiae} has thus supported the observations made earlier with mammalian cells. It has also substantiated the fact that the consensus sequence for PKA dependent phosphorylation in \textit{S. cerevisiae} DPMS is indeed functionally active. Enhancing the initial rate of the transferase activity with \textit{in vitro} phosphorylated recombinant enzyme was not due to a change in the $K_m$ for GDP-mannose but because of an increase in the $V_{\text{max}}$. This increase in enzyme activity is corroborated with the enzyme turnover ($k_{\text{cat}}$) as well as its catalytic efficiency ($k_{\text{cat}}/K_m$). DPMS also exhibited elevated level of activity when \textit{in vitro} phosphorylated enzyme was analyzed in the presence of increasing concentrations of Dol-P (data not shown). These results have strongly supported that increased synthase activity in phosphorylated DPMS is not due to a mere change in the affinity for the substrate, but most likely due to a conformational change of the enzyme. Future analysis of the DPMS protein structure by x-ray crystallography is expected to clarify this hypothesis.

Sequencing of yeast genome has detected 113 conventional protein kinases in budding yeast. This corresponds to ~2% of the total genes and more than 60% of these protein kinases have either known or suspected function (51). Therefore, the presence of a consensus sequence (YRRVIS$^{141}\text{ST}$) in Dol-P-Man synthase from \textit{S. cerevisiae} where serine-141 is a target for PKA phosphorylation (17) is reasonable. \textit{In vitro} phosphorylation of the Dol-P-Man synthase with
gamma-[\textsuperscript{32}P] ATP followed by SDS-PAGE and autoradiographic analysis as well as by immunoblotting with anti-phosphoserine antibody have supported the phosphoprotein nature of the yeast DPMS. To confirm that serine-141 is the target for PKA phosphorylation, and is responsible for enhanced activity of the phosphorylated DPMS, we have substituted the serine residue with alanine by PCR site-directed mutagenesis. Restriction enzyme analysis as well as the DNA sequencing has confirmed the S141A substitution in the Dpm1 gene. It is important to note that the S141A mutant exhibits a small increase in basal DPMS activity compared with that of the wild type. This may be due to the \textit{in vitro} assay condition, or due to a change in the protein conformation in this mutant, or it may be a combination of the two and can only be explained by x-ray crystallographic studies in the future. \textit{In vitro} phosphorylated S141A mutant DPMS is slightly more active (less than 2-fold) than its wild type counter part. If serine-141 were the only target for PKA-mediated phosphorylation, then one would expect no increase in enzyme activity in S141A DPMS mutant following phosphorylation. The only convincing argument at this point could be the presence of serine-142 in the DPMS sequence. If there is a change in the protein conformation due to a S141A mutation then it may be overriding the significance of serine-141 mutation, and making serine-142 accessible to PKA with a lower affinity. On the other hand, serine-142 could play as an alternative phosphorylation site in the absence of serine-141 to respond to cAMP-related stimuli. Thus, we conclude that serine-141 is the primary target for PKA-mediated phosphorylation of DPMS while responding to cAMP signaling. This valuable information is a step forward to understand the fundamentals of the closely related DPMS family of proteins, and the development of CDGS.

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Table I

Activation of Recombinant *S. cerevisiae* Dol-P-Man Synthase After In Vitro Protein Phosphorylation by cAMP-Dependent Protein Kinase

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Dol-P-Man (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.25 ± 0.27</td>
</tr>
<tr>
<td>After <em>in vitro</em> Phosphorylation</td>
<td>22.61 ± 0.46 *</td>
</tr>
</tbody>
</table>

Recombinant Dol-P-Man synthase was phosphorylated *in vitro* by incubating with a catalytic subunit from cAMP-dependent protein kinase (12 units of kinase per 30 µg of DPMS) at 30°C for 20 min in the presence of 10 mM NaF, 10 mM MgCl₂ and 200 µM ATP. The synthase activity was assayed by incubating at 37°C for 2 min in 30 mM Hepes, pH 7.0 buffer containing 150 µM EDTA, 30 µM DTT, 3% glycerol, 90 mM NaCl, 0.25% Triton X-100, 10 mM MnCl₂, 10 µg Dol-P, 1% Me₂SO and 0.125 µM GDP-*[^14]C* mannose (Sp. Act. 318 cpm/pmol) in a total volume of 100 µl as described in Materials and Methods. Results are expressed as mean ± SEM done in duplicates and analyzed by the student ‘t’ test. *p = 0.05.
Table II

Kinetic Constants of Recombinant Dol-P-Man Synthase Before and After Phosphorylation by cAMP-Dependent Protein Kinase

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>$K_m$ (M)</th>
<th>$V_{max}$ (nmol/min/mg Protein)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$1.2 \times 10^{-6}$</td>
<td>25.1</td>
<td>12.1</td>
<td>$1 \times 10^7$</td>
</tr>
<tr>
<td>After in vitro Phosphorylation</td>
<td>$1.1 \times 10^{-6}$</td>
<td>146.7</td>
<td>70.9</td>
<td>$6.4 \times 10^7$</td>
</tr>
</tbody>
</table>

Recombinant Dol-P-Man synthase was assayed as described in Table I but varying the GDP-mannose concentrations (0.0 - 5.0 µM) and incubating at 37º for 30 seconds.
Figure Legends

**Figure 1:** *Time Course of Recombinant Dol-P-Man Synthase Activity:* Enzymatic transfer of mannose from GDP-mannose to Dol-P was measured by incubating the control or *in vitro* phosphorylated enzyme at 37°C for 0 - 5 minutes in 30 mM Hepes, pH 7.0 buffer containing 150 µM EDTA, 30 µM DTT, 3% glycerol, 90 mM NaCl, 0.25% Triton X-100, 10 mM MnCl₂, 10 µg Dol-P, 1% Me₂SO and 0.125 µM GDP-[¹⁴C] mannose (Sp. Act. 318 cpm/pmol) in a total volume of 100 µl. Dol-P-Man was extracted and quantified in a liquid scintillation spectrometer. The results are the average from two determinations. ○---○, Control; •---•, *in vitro* Phosphorylated.

**Figure 2:** *Dependence of GDP-mannose Concentration on the Recombinant Dol-P-Man Synthase Activity:* Control and *in vitro* phosphorylated Dol-P-Man synthase activity was assayed by incubating at 37°C for 30 seconds as described in the Materials and Methods but in the presence of 0.125 - 5.0 µM GDP-[¹⁴C]-mannose (Sp. Act. 318 cpm/pmol). (a) v v/s [S] plot; (b) Lineweaver-Burk plot (1/v v/s 1/[S]). ○---○, Control; •---•, *in vitro* Phosphorylated.

**Figure 3:** *Autoradiographic Detection of [³²P]-labeled Dol-P-Man Synthase:* Recombinant Dol-P-Man synthase was phosphorylated *in vitro* by incubating with a catalytic subunit from cAMP-dependent protein kinase (12 units per 30 µg DPMS protein) at 30°C for 20 minutes in the presence of 30 mM Hesper, pH 7.0 buffer containing 10 mM NaF, 10 mM MgCl₂, and 5 µCi of gamma-[³²P] ATP. Control had everything except the cAMP-dependent protein kinase. [³²P]-labeled Dol-P-Man
synthase was immunoprecipitated with a mouse monoclonal antibody to yeast DPMS (1:1,000 dilution) for 3 h at 4°C followed by 3 h with 50 µg/ml of Protein A-sepharose CL-4B (30mg/ml) in the presence of aprotinin (1 µg/ml), PMSF (1 mM), TPCK (200 µM), soybean trypsin inhibitor (1µg/ml) and leupeptin (1 µM). The protein A-Sepharose:antigen-antibody complexes were washed twice with 200 µl of NET buffer, twice with 200 µl of washing buffer, and once with PBS, pH 7.4. The immune complexes were released by boiling at 100°C for 5 min and DPMS was separated on a 12% SDS-PAGE for 1 h at 190 volts in a Mini Protein Cell (Bio-Rad Laboratories). The gel was fixed in methanol-acetic-water (5:1:5, v/v/v), treated with Amplify™, dried and exposed to an X-ray film. Lane 1 = 14C-methylated protein markers; Lane 2 = [32P]Dol-P-Man synthase.

**Figure 4:** Immunoblotting of Recombinant Dol-P-Man synthase with anti-DPMS and anti-Phosphoserine Antibodies: Dol-P-Man synthase was phosphorylated *in vitro* and separated on a 12% SDS-PAGE as described in Figure 3. The protein was transblotted onto a 0.2 µm nitrocellulose membrane for 1 h at 100 volts using 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3. The blots were blocked overnight at 15°C in a blocking solution (5% non-fat dry milk in TTBS) and incubated subsequently with (a) anti-Dol-P-Man synthase antibody (diluted 1:2,000 in blocking solution), and (b) and (c) biotin-conjugated anti-phosphoserine antibody (diluted 1:2,000 in blocking solution) for 3 h at room temperature. The membranes were rinsed twice with TTBS, washed with one change of TTBS for 15 min and then twice for 5 min each at room temperature. The blot ‘a’ was treated with HRP-conjugated sheep anti-mouse IgG (1:2,000 dilution) to detect the Dol-P-Man synthase; and the blot ‘b’ and ‘c’ were treated
with streptavidin-conjugated sheep anti-mouse IgG (1:2,000 dilution) to detect the phosphoserine as well as the biotinylated-molecular weight markers. The blots were developed with ECL kit according to the manufacturer’s instructions. (a) Immunoblot for Dol-P-Man synthase: Lane 1 = biotinylated molecular weight markers; Lane 2 = Recombinant Dol-P-Man synthase detected with anti-DPMS antibody; (b) Immunoblot for Phosphoserine: Lane 1 = \textit{in vitro} phosphorylated recombinant DPMS; Lane 2 = \textit{in vitro} phosphorylated \textit{E. coli} cell extracts from IPTG-induced cells; Lane 3 = \textit{in vitro} phosphorylated \textit{E. coli} cell extracts from IPTG-uninduced cells; (c) Immunoblot for Phosphoserine: Lane 1 = mol. Wt marker; Lanes 2 and 3 = IPTG-uninduced \textit{E. coli} extracts carrying wild type DPMS gene; Lanes 4 and 5 = IPTG-uninduced \textit{E. coli} extracts carrying S141A DPMS mutant gene; Lane 6 = IPTG-induced \textit{E. coli} extracts carrying wild type DPMS gene; Lane 7 = IPTG-induced \textit{E. coli} extracts carrying S141A DPMS mutant gene.

\textbf{Figure 5:} \textit{Expression of Wild Type and S141A Dol-P-Man Synthase Mutant:} (a) \textbf{Analysis by SDS-PAGE:} 1 ml aliquots from uninduced and induced \textit{E. coli} cultures were pelleted by centrifugation (14,000 rpm for 30 seconds in a microcentrifuge) and boiled for 5 minutes in 1X sample buffer. 10 µl aliquots representing 200 µl of cells were applied to a 12% SDS-PAGE and elecrophoresed at 180 volts for 45 minutes. The gel was stained with 0.1% Coomassie Blue (250R) to visualize the protein bands. Lane M = molecular weight markers; Lane U (WT) = wild type DPMS before induction; Lane I (WT) = wild type DPMS after induction; Lane U (S141A) = S141A mutant DPMS before induction; Lane I (S141A) = S141A mutant DPMS after induction. (b) \textbf{Analysis by immunoblotting:} The wild type
and the S141A mutant DPMS proteins were separated on a 12% SDS-PAGE as mentioned above and transblotted onto a 0.2 µm nitrocellulose membrane. The blot was treated with the blocking solution overnight at 15°C and subsequently with yeast anti-Dol-P-Man synthase monoclonal antibody (1:2,000 dilution in blocking solution) for 3 h at room temperature. The membrane was rinsed twice with TTBS, washed with one change of TTBS for 15 minutes and twice for 5 minutes each at room temperature. The blot was then treated with HRP-conjugated sheep anti-mouse IgG (1:2,000 dilution) to detect the Dol-P-Man synthase and streptavidin-conjugated sheep anti-mouse IgG (1:2,000 dilution) to detect the biotinylated molecular weight markers. The blot was developed with the ECL kit and exposed to a Hyperfilm until the desired intensity was achieved. Lane M = biotinylated molecular weight markers; Lane WT = wild type DPMS; Lane S141A = phosphorylation-site deficient DPMS mutant.

**Figure 6:**

*Dol-P-Man Synthase Activity in S141A Mutant After in vitro Phosphorylation by cAMP-Dependent Protein Kinase:* Equal amounts of wild type and S141A DPMS mutant protein were phosphorylated *in vitro* and the activity of the phosphorylated enzymes were compared. Details of the phosphorylation conditions are described in **Table 1**. The results are an average from three determinations. (a) DPMS specific activity; (b) percentage changes of the DPMS specific activity after *in vitro* phosphorylation relative to the wild type DPMS. WT = wild type; S141A = phosphorylation-site deficient mutant.
Figure 1
Figure 5

(a)

(b)