Scanning Alanine Mutagenesis of the CDP-alcohol Phosphotransferase Motif of *Saccharomyces cerevisiae* Cholinephosphotransferase

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**Abstract**

Cholinephosphotransferase (EC 2.7.8.2) catalyzes the formation of a phosphoester bond via the transfer of a phosphocholine moiety from CDP-choline to diacylglycerol forming phosphatidylcholine and releasing CMP. A motif, Asp113-Gly114-Ala117-Arg118-Gly127-Asp131-Ala135, located within the CDP-choline binding region of *Saccharomyces cerevisiae* cholinephosphotransferase (CPT1) is completely conserved in all members of this family. The recombinant enzyme was exploited to delineate the CDP-choline binding site. Analysis of the alignment of sequences revealed a completely conserved motif, Asp-Gly-X-Ala-Arg-Gly-X-Asp, termed the CDP-alcohol phosphotransferase motif. Data base searches revealed this motif was diagnostic for the reaction type catalyzed by each. To lend credence to this prediction, and to provide insight into catalytic mechanism, scanning alanine mutagenesis of the conserved residues within the CDP-alcohol phosphotransferase was performed. Enzyme activity was assessed in *vitro* using a mixed micelle enzyme assay and in *vivo* by determining the ability of the mutant enzymes to restore phosphatidylycholine synthesis from radiolabeled choline in an *S. cerevisiae* strain devoid of endogenous cholinephosphotransferase activity. Ala117 and Arg118 displayed reduced enzyme activity, and Asp113 displayed wild type activity. The analysis described is the first molecular characterization of a CDP-alcohol phosphotransferase motif and results predict a catalytic role utilizing a general base reaction proceeding through Asp113 or Asp131 via a direct nucleophilic attack of the hydroxyl of diacylglycerol on the phosphoester bond of CDP-choline that does not proceed via an enzyme bound intermediate. Residues Ala117 and Arg118 do not participate directly in catalysis but are likely involved in substrate binding or positioning with Arg118 predicted to associate with a phosphate moiety of CDP-choline.

Cholinephosphotransferase catalyzes the transfer of phosphocholine from CDP-choline to diacylglycerol (DAG) thus forming phosphatidylcholine (PtdCho) and CMP. As the final step of the Kennedy pathway (1, 2) cholinephosphotransferase identifies both the site of *de novo* PtdCho synthesis as well as the site from which PtdCho is transported to other organelles within the cell or assembled with proteins and other lipids for export from the cell during the genesis of lung surfactant, bile, and lipoproteins (3–5). Two genes coding for cholinephosphotransferase activities exist in *Saccharomyces cerevisiae*, CPT1, which encodes a cholinephosphotransferase (6), and EPT1, which codes for a dual specificity choline/ethanolaminophosphotransferase (7). In *vitro*, Cpt1p and Ept1p contribute equally to measurable cholinephosphotransferase activity (8); however, in *vivo* metabolic labeling analysis revealed that Cpt1p is responsible for 95% of the PtdCho-synthesizing cholinephosphotransferase activity (9); however, in *vivo* metabolic labeling analysis revealed that Cpt1p is responsible for 95% of the PtdCho-synthesizing cholinephosphotransferase activity (9); however, in *vivo* metabolic labeling analysis revealed that Cpt1p is responsible for 95% of the PtdCho-synthesizing cholinephosphotransferase activity (9); however, in *vivo* metabolic labeling analysis revealed that Cpt1p is responsible for 95% of the PtdCho-synthesizing cholinephosphotransferase activity (9); however, in *vivo* metabolic labeling analysis revealed that Cpt1p is responsible for 95% of the PtdCho-synthesizing cholinephosphotransferase activity (9); however, in *vivo* metabolic labeling analysis revealed that Cpt1p is responsible for 95% of the PtdCho-synthesizing cholinephosphotransferase activity (9). The difference in CDP-alcohol specificity between the parental Cpt1p and Ept1p enzymes was exploited to delineate the CDP-choline binding site. A region encompassing the first soluble loop, residues 79–186 of Cpt1p, was pinpointed. Data base searches for known proteins with homology to the CDP-choline binding region of Cpt1p identified sequences within *S. cerevisiae* ethanolaminophosphotransferase (EPT1) (7), phosphatidylinositol (PtdIns) synthase (PIS1) (12), and phosphatidylserine (PtdSer) synthase (PSS1/CHO1) (13); prokaryotic phosphatidylglycerol (PtdGro) phosphosphate and PtdSer (Gram-positive only) synthases (14, 15); soybean aminolinocholeophosphotransferase (16); and rat PtdIns synthase (17). Each of these enzymes catalyzes the synthesis of a phospholipid by the displacement of CMP from a CDP-alcohol by a second alcohol to form a phosphoester bond. Alignment of the sequences within each of the above enzymes revealed a completely conserved motif, Asp-Gly-X-Ala-Arg-Gly-X-Asp, termed the CDP-alcohol phosphotransferase motif. Data base searches revealed this motif was specific to the above enzymes and hence is predicted to be diagnostic for the reaction type catalyzed by each. To lend credence to this prediction, and to provide insight into catalytic mechanism, scanning alanine mutagenesis of the conserved residues within the CDP-alcohol phosphotransferase

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The abbreviations used are: DAG, diacylglycerol; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; PtdGro, phosphatidylglycerol; HA, hemagglutinin; mA, monoclonal antibody; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; CPT1, cholinephosphotransferase gene of *S. cerevisiae*; Cpt1p, cholinephosphotransferase protein of *S. cerevisiae*; EPT1, ethanolaminophosphotransferase gene of *S. cerevisiae*. © 1998 by The American Society for Biochemistry and Molecular Biology, Inc.
motif of *S. cerevisiae* cholinephosphotransferase (CPT1) was performed.

**EXPERIMENTAL PROCEDURES**

**Materials**—[α-32P]dATP (3000 Ci/mmol) and [methyl-14C]choline were products of NEN Life Science Products. [methyl-14C]CDP-choline was purchased from American Radiolabeled Chemicals. Custom oligonucleotides were purchased from Life Technologies, Inc. Dideoxy sequencing was performed utilizing the T7 sequencing kit (Amersham Pharmacia Biotech). Lipids were purchased from Avanti Polar Lipids. Sequencing was performed utilizing the T7 sequencing kit (Amersham). Nucleotides were purchased from Life Technologies, Inc. Dideoxy sequencing was performed utilizing the T7 sequencing kit (Amersham Pharmacia Biotech). Lipids were purchased from Avanti Polar Lipids. Sequencing was performed utilizing the T7 sequencing kit (Amersham). Nucleotides were purchased from Life Technologies, Inc. Dideoxy sequencing was performed utilizing the T7 sequencing kit (Amersham Pharmacia Biotech). Lipids were purchased from Avanti Polar Lipids. Sequencing was performed utilizing the T7 sequencing kit (Amersham Pharmacia Biotech). Lipids were purchased from Avanti Polar Lipids.

**Site-directed Mutagenesis—**Plasmid pH150 (6) contains the CPT1 gene in the high copy plasmid YEp352 (18) and was used as the template for all mutagenesis reactions. The T4 DNA polymerase-directed MORPH plasmid DNA mutagenesis protocol (5) was used with the appropriate mutagenic oligonucleotides (Table I) as directed by the manufacturer. All mutations were confirmed by DNA sequencing. Plasmids were routinely propagated in DH5α (Amersham Pharmacia Biotech) with the appropriate mutagenic oligonucleotides (Table I) as directed.

**PtdCho Biosynthesis—**Yeast strain HJ091 (a ura3-52 his3-1 leu2-3, 112 trp1-289 cpl1-LEU2 cpl1-1) was utilized in all studies. HJ091 is devoid of detectable cholinephosphotransferase activity (10, 11). Microsomal membranes were prepared from HJ091 grown at 30 °C to mid-log phase in synthetic dextrose media containing the appropriate nutritional supplements to ensure plasmid maintenance (19). Cholinephosphotransferase activity was assessed with 20 μM MgCl2, as cation cofactor, 10 mol % PtdChol (egg) as phospholipid cofactor, and 10 mol % dipalmityl-1,2-dioleoylglycerol (d16:1DAG) and 500 μM [14C]choline (500,000 dpm/μL) as substrates in a Triton X-100 based mixed micelle assay (19). The CPT1p CDP-alcohol phosphotransferase Mutants—Scanning alanine site-directed mutagenesis of the CDP-alcohol phosphotransferase motif [Asp113>Gly114→Ala] (29, 30) was performed using the method of Lowry et al. (20) using bovine serum albumin as standard. DAG was prepared from PtdCho by phospholipase C digestion, and yeast extract was used as the substrate for the method of Step and Shapi (21). Phospholipid phosphorus was determined by the method of Ames and Dubin (22).

**RESULTS**

**Enzyme Activity of the Cpt1p CDP-alcohol Phosphotransferase Mutants—**Scanning alanine site-directed mutagenesis of the CDP-alcohol phosphotransferase motif [Asp113>Gly114→Ala] (29), [Asp113→Gly] (20), and [Asp113→Glu] (21) was performed using the method of Lowry et al. (20) using bovine serum albumin as standard. DAG was prepared from PtdCho by phospholipase C digestion, and yeast extract was used as the substrate for the method of Step and Shapi (21). Phospholipid phosphorus was determined by the method of Ames and Dubin (22).

**Protein and Lipid Determinations—**Protein was determined by the method of Lowry et al. (20) using bovine serum albumin as standard. DAG was prepared from PtdCho by phospholipase C digestion, and yeast extract was used as the substrate for the method of Step and Shapi (21). Phospholipid phosphorus was determined by the method of Ames and Dubin (22).

**Immunodetection—**The CPT1 gene and site-directed mutants were subcloned from YEp352 [URA3, 2 μ ori] to pRS426 [URA3, 2 μ ori] to facilitate the elimination of a HindIII site within the multicloning site. A 3 × repeat of the HA epitope was amplified by polymerase chain reaction from the plasmid pGTEST (gift from Stephen Garrett, Duke University Medical Center) with HindIII sites added to the ends of each primer. The amplified 3 × HA epitope was subcloned into a unique HindIII site within the CPT1 coding region corresponding to amino acids Tyr270 and Leu279. The insert was sequenced to ensure polymerase frame and fidelity. HJ091 cells were transformed with the constructs and microsomal membranes were isolated. Identical (10 μg) amounts of microsomal protein were incubated with equal volumes of 2 × SDS sample buffer (50 mM Tris-HCl (pH 6.8), 10% glycerol (v/v), 2% SDS (w/v), 5% 2-mercaptoethanol (v/v), 0.05% bromophenol blue (w/v)) at 37 °C for 20 min. Proteins were separated by SDS-PAGE (4%) stacking gel, 12.5% resolving gel), and transferred to polyvinylidene fluoride membrane utilizing the Bio-Rad mini gel transfer apparatus at 30 volts for 1.5 h in 48 mM Tris, 30 mM glycine, 0.1% SDS, 20% methanol (v/v) transfer buffer. The membrane was blocked with phosphate-buffered saline (PBS) containing 5% skim milk powder and 0.05% Tween 20 for 1 h; washed three times with PBS, 0.05% Tween 20; incubated for 1 h in PBS, 0.05% Tween 20 with HA antibody (1:2,000); washed three times with PBS, 0.05% Tween 20; incubated for 1 h in PBS, 0.05% Tween 20 with goat anti-mouse Ab coupled to horseradish peroxidase (1:10,000); washed three times with PBS, 0.05% Tween 20; and signal was detected using the ECL (Amersham) method as per the manufacturer’s instructions.

**Enzyme Activity of the Cpt1p CDP-alcohol Phosphotransferase Mutants—**Scanning alanine site-directed mutagenesis of the CDP-alcohol phosphotransferase motif [Asp113>Gly114→Ala] (29), [Asp113→Gly] (20), and [Asp113→Glu] (21) was performed using the method of Lowry et al. (20) using bovine serum albumin as standard. DAG was prepared from PtdCho by phospholipase C digestion, and yeast extract was used as the substrate for the method of Step and Shapi (21). Phospholipid phosphorus was determined by the method of Ames and Dubin (22).

**Specific enzyme activities were determined using a Triton X-100 based mixed micelle assay (8, 10, 23) under optimal reaction conditions for the wild type enzyme (Table I). Conversion of Asp113 to Ala did not appreciably effect enzyme activity.
activity; however, Ala117 to Gly, and Arg118 to Ala replacements decreased activity to 17.8% and 10.1% wild type levels. Separate conversion of Gly114 to Ala as well as each of the final three amino acids within the motif, Gly127, Asp131, and Asp135, to Ala eliminated detectable cholinephosphotransferase activity. Increasing the sensitivity of the assay by increasing the specific radioactivity of the substrate 8-fold coupled with 4-fold additional protein, a 32-fold total increase in activity detectability over standard assay conditions, did not result in radiolabeled PtdCho production, indicating these mutations effectively eliminated enzyme activity.

**In Vivo Analysis of the Cpt1p CDP-alcohol Phosphotransferase Mutants**—To corroborate the above in vitro assessment of the ability of the various CDP-alcohol phosphotransferase motif site-directed mutants to confer cholinephosphotransferase activity, each mutant was expressed in *S. cerevisiae* cells devoid of cholinephosphotransferase and their capacity to incorporate radiolabeled choline into PtdCho was determined (Fig. 2). Since the rate-limiting step for PtdCho synthesis is normally at the level of phosphocholine cytidylyltransferase, alterations in cholinephosphotransferase activity do not generally affect the ability of cells to incorporate radiolabeled choline into PtdCho (9, 24, 26, 27). In addition, the metabolic labeling protocol is much more sensitive than the in vitro enzyme assay and does not rely on the ability of the various mutant enzymes to survive cell disruption and subcellular fractionation and hence provides a second level of confidence for determining if mutants with undetectable cholinephosphotransferase activity in vitro are indeed devoid of enzyme activity. The enzymatically active Asp113 to Ala, Ala117 to Gly, and Arg118 to Ala mutants incorporated labeled choline into PtdCho at a level similar to that of cells carrying the parental Cpt1p protein.
Gly\textsuperscript{114} to Ala, Gly\textsuperscript{127} to Ala, Asp\textsuperscript{131} to Ala, and Asp\textsuperscript{135} to Ala mutants, each of which was inactive \textit{in vitro}, were unable to incorporate radiolabeled choline into PtdCho (Fig. 2B). In agreement with a metabolic block at the level of cholinephosphotransferase in the \textit{S. cerevisiae} cells used in this study (\textit{cpt1}:\textit{LEU2} \textit{cpt1}'), this yeast strain (and each of the inactive \textit{Cpt1p} mutants) accumulated CDP-choline (Fig. 2C), while the wild type cells (and each of the mutants with detectable cholinephosphotransferase activity \textit{in vitro}) did not accumulate CDP-choline due to its successful conversion to PtdCho (Fig. 2, B and C).

**Kinetic Analysis of the \textit{Cpt1p} CDP-alcohol Phosphotransferase Mutants**—As a first step in determining catalytic mechanisms for cholinephosphotransferase, a kinetic analysis of each of the enzymatically active mutants was performed and compared with the parental enzyme. The mixed micelle assay employed revealed saturation kinetics for both substrates when the other was in excess (data not shown) implying surface lability. Conditions wild type \textit{Cpt1p} displayed an apparent \(K_m\) for CDP-choline 2.5-fold and for DAG 2.8-fold over those of the parental enzyme. The Arg\textsuperscript{218} to Ala mutation resulted in increased apparent \(K_m\) values of 3.8-fold for CDP-choline and 3.1-fold for DAG. Predicted \(V_{\text{max}}\) values were 1.01 and 1.21 nmol min\(^{-1}\) mg\(^{-1}\) for the \textit{Ala\textsuperscript{117}} to Gly mutant, and 0.60 and 0.55 nmol min\(^{-1}\) mg\(^{-1}\) for the Arg\textsuperscript{218} to Ala mutant (Tables III and IV).

**Immunodetection of Parental and Mutant Cholinephosphotransferases**—In this study, parental and mutant \textit{CPT1} genes were expressed from high copy (URA3, 2 \(\mu\) ori) plasmids using the endogenous \textit{CPT1} promoter. Western blot analysis was performed to ensure that the various mutations created within \textit{Cpt1p} (with activities significantly different from wild type) were not affecting \textit{Cpt1p} levels. A 3 \(\times\) repeat of the HA epitope was inserted into the coding region of the parental and site-directed mutant genes at a site between amino acids Tyr\textsuperscript{28} and Leu\textsuperscript{29}. Plasmids were transformed into \textit{HJ091} cells and microsomal membrane proteins were subjected to SDS-PAGE and Western blot analysis using mAb specific for the HA epitope (Fig. 3). The HA mAb recognized a protein of the expected size for \textit{Cpt1p}+3 \(\times\) HA epitope (49 kDa) that was absent in cells expressing \textit{Cpt1p} that did not contain the 3 \(\times\) HA epitope, demonstrating that the mAb was specific for epitope tagged \textit{Cpt1p} proteins (the increased size of \textit{Ala}\textsuperscript{117} is a function of the cloning strategy utilized that resulted in a 6 \(\times\) insertion of the \textit{HA} epitope). Parental and mutant \textit{Cpt1p} proteins \textit{Ala}\textsuperscript{117} to Gly, Arg\textsuperscript{218} to Ala, Gly\textsuperscript{127} to Ala, Asp\textsuperscript{131} to Ala, and Asp\textsuperscript{135} to Ala were present in similar amounts (Fig. 3) confirming that the decreased cholinephosphotransferase enzyme activity associated with the amino acid substitutions of these residues was due to altered kinetic properties of the mutant proteins and not due to decreased protein levels. No \textit{Cpt1p} protein was detected for the Gly\textsuperscript{114} to Ala mutant, indicating that the absence of cholinephosphotransferase activity associated with this amino acid substitution was due to increased protein lability.

**DISCUSSION**

Scanning alanine site-directed mutagenesis of the conserved amino acid residues within the CDP-alcohol phosphotransferase motif (Asp\textsuperscript{113}–Gly\textsuperscript{114}–X\textsuperscript{115}–Asp\textsuperscript{117}–Arg\textsuperscript{118}–X\textsuperscript{119}–Gly\textsuperscript{122}–X\textsuperscript{123}–Asp\textsuperscript{131}–X\textsuperscript{132}–Asp\textsuperscript{135}) of \textit{S. cerevisiae} cholinephosphotransferase was performed to lend credence to the prediction that this motif is diagnostic for the reaction catalyzed and to provide the first molecular investigation of the catalytic mechanism used by this class of enzymes which to date include: \textit{S. cerevisiae} cholinephosphotransferase, ethanolaminephosphotransferase, PtdSer

| \textbf{Cpt1p mutation} | \textbf{\(K_m\) (app) \(\mu\text{M}\)} | \textbf{\(V_{\text{max}}\) (app) \(\text{nmol min}^{-1} \text{mg}^{-1}\)} |
|--------------------------|-----------------|-----------------|
| Wild type                | 2.86            | 1.01            |
| Asp\textsuperscript{113} → Ala | 6.24            | 1.01            |
| Ala\textsuperscript{117} → Gly | 6.48            | 1.01            |
| Arg\textsuperscript{118} → Ala | 7.00            | 6.48            |

| \textbf{Cpt1p mutation} | \textbf{\(K_m\) (app) \(\text{mol} \text{\%}\)} | \textbf{\(V_{\text{max}}\) (app) \(\text{nmol min}^{-1} \text{mg}^{-1}\)} |
|--------------------------|-----------------|-----------------|
| Wild type                | 2.86            | 1.01            |
| Asp\textsuperscript{113} → Ala | 6.24            | 1.01            |
| Ala\textsuperscript{117} → Gly | 6.48            | 1.01            |
| Arg\textsuperscript{118} → Ala | 7.00            | 6.48            |

**TABLE III**

**Effect of CDP-choline on the kinetics of \textit{Cpt1p} CDP-alcohol phosphotransferase motif mutants**

**TABLE IV**

**Effect of diacylglycerol on the kinetics of \textit{Cpt1p} CDP-alcohol phosphotransferase motif mutants**

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**Fig. 3. Immunodetection of parental and site-directed mutants of \textit{Cpt1p}**. Microsomal membranes were isolated from \textit{HJ091} cells expressing parental and mutant forms of \textit{3\(\times\) HA epitope-tagged Cpt1p}. Equivalent amounts of microsomal protein were subjected to SDS-PAGE and analyzed by Western blot utilizing mAb specific for the HA epitope as described under "Experimental Procedures."
synthase, PtdIns synthase, prokaryotic PtdSer synthases, PtdGro phosphate synthases, and rat PtdIns synthase. Each of these enzymes catalyzes the formation of a phospholipid via the displacement of CMP from a CDP-alcohol by a second alcohol with concomitant formation of a phosphoester bond. The ability of each mutant to catalyze the cholinephosphotransferase reaction was assessed in vitro using a mixed micelle enzyme assay and in vivo by determining the capacity of each mutant to incorporate radiolabeled choline into PtdCho in a S. cerevisiae strain containing null mutations in its cholinephosphotransferase genes.

One mutant, Asp\textsuperscript{113} to Ala, displayed wild type characteristics both in vitro and in vivo. This is intriguing in that this, the first Asp residue within the CDP-alcohol phosphotransferase motif, is completely conserved over a wide range of species and hence has not drifted evolutionarily implying it is essential; however, its function is not apparent from the above analyses. Two mutants, A\textsuperscript{117} to G and B\textsuperscript{118} to A, displayed reduced in vitro catalytic activity due to 5.0–10.4-fold decreased V\textsubscript{max}(app) and 2.5–3.8-fold increased K\textsubscript{m}(app) values for both CDP-choline and DAG. These results imply Ala\textsuperscript{117} and Arg\textsuperscript{118} play a role in substrate binding or positioning with Arg\textsuperscript{118} predicted to be coordinated with one of the phosphate groups within the CDP-alcohol. Interestingly, in vivo the Ala\textsuperscript{117} to Gly and Arg\textsuperscript{118} to Ala mutants incorporated radiolabeled choline into PtdCho in cholinephosphotransferase null cells at levels similar to that of the parental enzyme. These data support two further conclusions: (i) the concentration of both CDP-choline and DAG at the intracellular site of cholinephosphotransferase must be sufficient to overcome the increase in K\textsubscript{m} (app) demonstrated by the Ala\textsuperscript{117} to Gly and Arg\textsuperscript{118} to Ala mutants, and (ii) cholinephosphotransferase is not rate-limiting, since large decreases in V\textsubscript{max}(app) did not affect the level with which labeled choline was incorporated into PtdCho. The latter conclusion is consistent with many other metabolic studies (24, 26). Mutations at Gly\textsuperscript{114}, Gly\textsuperscript{127}, Asp\textsuperscript{131}, and Asp\textsuperscript{135}, completely ablated detectable activity both in vitro and in vivo. The absence of cholinephosphotransferase activity in cells expressing Cpt1p carrying a Gly\textsuperscript{114} to Ala substitution correlated with an absence of detectable protein, indicating this residue is required for protein stability or folding. Since Gly residues do not possess a functional group, the lack of activity in the Gly\textsuperscript{127} to Ala mutant suggests a steric role within the active site. The elimination of cholinephosphotransferase activity by mutating either Asp\textsuperscript{131} or Asp\textsuperscript{135} (the last two residues within the motif) to Ala implies one of these is the catalytic residue.

Several members of the CDP-alcohol phosphotransferase motif family of enzymes have been subjected to kinetic analyses; pure enzyme preparations of S. cerevisiae PtdSer synthase and E. coli PtdGro phosphate synthase, as well as microsomal mammalian cholinephosphotransferase, predicted sequential bi-bi reaction mechanisms (28–31). An in depth kinetic analysis of purified E. coli PtdGro phosphate synthase was consistent with a reaction mechanism that did not utilize an enzyme bound intermediate. A general acid-catalyzed reaction utilizing an Asp residue at its catalytic center would favor an enzyme bound intermediate, while a general base reaction would not, hence, CDP-alcohol phosphotransferases most likely utilize general base catalysis via nucleophilic attack of the hydroxyl group of the free alcohol directly on the phosphoester bond of the CDP-alcohol through one of the final two Asp residues within the motif without passing through an enzyme-bound intermediate.

From the above results it is clear that the CDP-alcohol phosphotransferase motif, Asp-Gly-(X)\textsubscript{2}-Ala-Arg-(X)\textsubscript{n}Gly-(X)\textsubscript{n}-Asp-(X)\textsubscript{n}Asp, is diagnostic for the reaction catalyzed. However, it should be noted that this motif is not essential for this reaction type. E. coli PtdSer synthase catalyzes the formation of PtdSer via formation of a phosphoester bond utilizing CDP-DAG and serine as substrates with subsequent release of CMP in a manner similar to that of the Bacillus subtilis and S. cerevisiae PtdSer synthases (31–33). The latter two possess the CDP-alcohol phosphotransferase motif while the E. coli enzyme possesses a separate motif, Hx(U)\textsubscript{2}DX\textsubscript{2}UUGO, that also appears capable of the formation of the identical phosphoester bond (34, 35). This second motif is also found in prokaryotic cardiolipin synthase, as well as enzymes that catalyze the hydrolysis of a phosphoester bond including phospholipase D and some nucleases. Studies of E. coli and S. cerevisiae PtdSer synthases provide two further lines of evidence that are consistent with two distinct motifs catalyzing the same reaction via different mechanisms: (i) kinetic analysis of E. coli PtdSer synthase predicted a ping-pong reaction type that utilized an enzyme-bound intermediate (31–33), a mechanism distinct from the sequential bi-bi reaction of CDP-alcohol phosphotransferase motif enzymes (28–31), and (ii) an isotopic exchange NMR analysis comparing the PtdSer synthase activities from E. coli and S. cerevisiae observed a retention of chirality of the β-phosphorus within the CDP-diacylglycerol substrate for the E. coli enzyme (consistent with a reaction mechanism proceeding via an enzyme-bound intermediate), while the S. cerevisiae enzyme displayed an inversion of chirality of the β-phosphorus (implying a single displacement mechanism) (31).

This study is the first molecular characterization of a CDP-alcohol phosphotransferase motif and results indicate the motif plays an intimate role in catalysis. Several lines of evidence point to this conclusion: (i) the CDP-alcohol phosphotransferase motif is completely conserved in enzymes that catalyze the same reaction type; (ii) the conservation is observed across a wide range evolutionary range implying the preserved residues are essential for enzyme function; (iii) FASTAPAT and Motif searches of nonredundant data bases revealed only the above enzymes indicating its specificity; (iv) mutations within specific residues of the S. cerevisiae cholinephosphotransferase CDP-alcohol phosphotransferase motif abolish or reduce activity. The integral membrane-bound nature of all of the members of the CDP-alcohol phosphotransferase motif enzymes has precluded their purification from most sources, including any mammalian cell type, thus many of their respective cDNAs have yet to be isolated. The verification of a conserved motif diagnostic for the reaction type catalyzed by each of these enzymes will allow for the rapid identification of cDNAs coding for these proteins from expressed sequence tag data bases. These new molecular tools will allow for a precise dissection of the many biological and pathophysiological roles currently postulated for each of these enzymes.

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