Phosphorylation of CTP Synthetase on Ser\textsuperscript{36}, Ser\textsuperscript{330}, Ser\textsuperscript{354}, and Ser\textsuperscript{454} Regulates the Levels of CTP and Phosphatidylcholine Synthesis in \textit{Saccharomyces cerevisiae}\textsuperscript{*}

Tae-Sik Park, Daniel J. O'Brien, and George M. Carman\textsuperscript{‡}

From the Department of Food Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, New Jersey 08901

The \textit{Saccharomyces cerevisiae} URA7-encoded CTP synthetase is phosphorylated and stimulated by protein kinase C. We examined the hypothesis that Ser\textsuperscript{36}, Ser\textsuperscript{330}, Ser\textsuperscript{354}, and Ser\textsuperscript{454}, contained in a protein kinase C sequence motif in CTP synthetase, were target sites for the kinase. Synthetic peptides containing a phosphorylation motif at these serine residues served as substrates for protein kinase C \textit{in vitro}. Ser\textsuperscript{36}→Ala (S36A, S330A, S354A, and S454A) mutations in CTP synthetase were constructed by site-directed mutagenesis and expressed normally in a \textit{ura7 ura8} double mutant that lacks CTP synthetase activity. The CTP synthetase activity in extracts from cells bearing the S36A, S354A, and S454A mutant enzymes was reduced when compared with cells bearing the wild type enzyme. Kinetic analysis of purified mutant enzymes showed that the S36A and S354A mutations caused a decrease in the \textit{V}_{\text{max}}\text{ of the reaction. This regulation could be attributed in part by the effects phosphorylation has on the nucleotide-dependent oligomerization of CTP synthetase. In contrast, CTP synthetase activity in cells bearing the S330A mutant enzyme was elevated, and kinetic analysis of purified enzyme showed that the S330A mutation caused an elevation in the \textit{V}_{\text{max}}\text{ of the reaction. In vitro data indicated that phosphorylation of CTP synthetase at Ser\textsuperscript{330} affected the phosphorylation of the enzyme at another site. The phosphorylation of CTP synthetase at Ser\textsuperscript{36}, Ser\textsuperscript{330}, Ser\textsuperscript{354}, and Ser\textsuperscript{454} residues was physiologically relevant. Cells bearing the S36A, S354A, and S454A mutations had reduced CTP levels, whereas cells with the S330A mutation had elevated CTP levels. The alterations in CTP levels correlated with the regulatory effects CTP has on the pathways responsible for the synthesis of the membrane phospholipid phosphatidylcholine.

In the yeast \textit{Saccharomyces cerevisiae} CTP synthetase catalyzes the ATP-dependent transfer of the amide nitrogen of glutamine to the C-4 position of UTP to form CTP (1, 2). ATP stimulates the reaction by accelerating the formation of a covalent glutaminyl enzyme catalytic intermediate (2–5). URA7 (6) and URA8 (7) are duplicate genes that code for CTP synthetase in \textit{S. cerevisiae}. The yeast CTP synthetase enzymes (6, 7) contain a conserved glutamine amide transfer domain (see Fig. 1) common to CTP synthetases from other organisms (8–16). The URA7-encoded CTP synthetase is more abundant than the URA8-encoded enzyme (17) and is responsible for the majority of the CTP synthesized \textit{in vivo} (7). Neither the URA7 nor the URA8 gene is essential as long as cells possess one functional CTP synthetase gene (6, 7). CTP synthetase is an indispensable enzyme because its reaction product CTP is essential for the synthesis of nucleic acids and membrane phospholipids (18). The importance of understanding the regulation of CTP synthetase is emphasized by the fact that unregulated levels of its activity is a common property of various cancers in humans (19–26).

The yeast CTP synthetase is regulated by genetic and biochemical mechanisms. Like many enzymes involved in macromolecular synthesis, CTP synthetase is regulated by growth phase. CTP synthetase mRNA and protein levels are highest in the exponential phase of growth and decline as cells enter the stationary phase (28). CTP synthetase activity is allosterically regulated by its substrates and product CTP. The enzyme exhibits positive cooperative kinetics with respect to UTP and ATP and negative cooperative kinetics with respect to glutamine and CTP (5, 28). The positive cooperative kinetics of the URA7-encoded enzyme with respect to UTP and ATP are due to the nucleotide-dependent oligomerization of an inactive dimeric form to an active tetrameric form of the enzyme (29). A major form of CTP synthetase regulation is mediated by CTP product inhibition (5, 28). CTP inhibits CTP synthetase activity by increasing the positive cooperativity of the enzyme for UTP (5). Amino acid residue Glu\textsuperscript{161} has been identified as being involved in this regulation (see Fig. 1) (30).

Phosphorylation is a major mechanism by which enzymes are regulated (31, 32), and indeed, the yeast CTP synthetase is regulated by phosphorylation. The URA7-encoded CTP synthetase is phosphorylated on multiple serine residues \textit{in vivo} (33). In vitro studies show that CTP synthetase is a substrate for protein kinases A (34) and C (33, 35). These phosphorylations result in the stimulation of CTP synthetase activity by a mechanism that increases catalytic turnover (33–35). In addition, phosphorylation facilitates the nucleotide-dependent tetramerization of the enzyme (29) and causes a decrease in the sensitivity of the enzyme to inhibition by CTP (34, 35). Ser\textsuperscript{454} has been identified as the target site for protein kinase A phosphorylation (see Fig. 1) (36). However, the site(s) of phosphorylation for protein kinase C is unknown. In this study we examined the hypothesis that amino acid residues Ser\textsuperscript{36}, Ser\textsuperscript{330}, Ser\textsuperscript{354}, and Ser\textsuperscript{454} within a protein kinase C phosphorylation motif in the URA7-encoded CTP synthetase are target sites of phosphorylation (Fig. 1). We showed that S36A, S330A, S354A, and S454A mutant CTP synthetase enzymes exhibited alter-
phoresis were purchased from New England Biolabs. Oligonucleotides
nuclease activity and the DNA size ladder used for agarose gel electro-
plies were purchased from Difco. Restriction endonucleases, modifying
site involved in CTP product inhibition, and the protein kinase A phosphorylation site in CTP synthetase. The Ser 36, Ser330, Ser354, and Ser 454
residues within a protein kinase C phosphorylation motif that were mutated to alanine residues are indicated. The numbers on the top of
the diagram denote the amino acid positions in the CTP synthetase protein.
ations in their catalytic properties and that cells carrying the mutant enzymes exhibited alterations in the cellular levels of
and in the synthesis of the membrane phospholipid PC.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were reagent grade. Growth medium sup-
pplies were purchased from Difeo. Restriction endonucleases, modifying
enzymes, and recombinant Vent DNA polymerase with 5’ and 3’ exo-
nuclease activity and the DNA size ladder used for agarose gel electro-
phoresis were purchased from New England Biolabs. Oligonucleotides
were prepared commercially by Genosys Biotechnologies, Inc. The
QuickChange site-directed mutagenesis kit was purchased from Strat-
gene. The Prism DyeDeoxy DNA sequencing kit was obtained from
Applied Biosystems. Nucleotides, 1-glutamine, 5-fluoroorotic acid, phen-
ylmethylsulfonyl fluoride, benzamidine, aprotinin, leupeptin, pepsta-
in, nitrocellulose paper, casein, and bovine serum albumin were pur-
chased from Sigma. Peptides were synthesized and purified commercially by Bio-Synthesis, Inc. Protein assay, electrophoresis re-
agents, and protein markers were purchased from Bio-Rad. Superose 6,
Mono Q, ECF Western blotting kit, and 2’, 3’-dideoxyxynucleotide triphosphates were purchased from Amersham Biosciences. IMMUNO-
catcher immunoprecipitation kit was purchased from CytoSignal Re-
search Products. Centricon-10 concentration filters were purchased from
Amicon. Phosphocellulose filter kits and dialysis cassettes were
purchased from Pierce. Radiochemicals were purchased from PerkinElmer Life Sciences. Scintillation counting supplies and acryl-
amide solutions were from National Diagnostics. Phospholipids were
from Avanti Polar Lipids. Silica Gel 60 thin-layer chromatography plates were purchased from EM Science.

Strains, Plasmids, and Growth Conditions—The strains and plas-
mids used in this work are listed in Table I. Wild type and mutant alleles of the
ura7-encoded CTP synthetase were expressed in the
ura7 ura8 double mutant strain SD195 (30). Growth of this strain is
dependent on a plasmid bearing either the URA7 or the URA8 gene
(30). Methods for growth and analysis of yeast were performed as described previously (37, 38). Yeast cultures were grown in complete
synthetic medium minus inositol (39) containing 2% glucose at 30 °C.
Plasmid maintenance and amplifications were performed in Esche-
richia coli strain DH5α. E. coli cells were grown in LB medium (1% Tryptone, 0.5% yeast extract, 1% NaCl (pH 7.4)) at 37 °C. Ampicillin
(100 μg/ml) was added to cultures carrying plasmids. Media were sup-
plemented with either 2% (yeast) or 1.5% (E. coli) agar for growth on
plates. Yeast cell numbers in liquid media were determined by micro-
scopic examination with a hemacytometer or spectrophotometrically at an
absorbance of 600 nm.

DNA Manipulations, Amplification of DNA by PCR, and DNA Se-
quencing—Plasmid DNA preparation, restriction enzyme digestion,
and DNA ligations were performed by standard methods (38). Trans-
formation of yeast (40, 41) and E. coli (38) were performed as described
previously. Conditions for PCR reactions were optimized as described
previously (42). DNA sequencing reactions were performed by the
dideoxy method using T7g DNA polymerase (38).

Constructions of Plasmids—The codons for Ser26, Ser130, Ser354, and
Ser454 in the ura7-encoded CTP synthetase were changed to alanine
codons by site-directed mutagenesis. The URA7allele (primers: 5’-CCC-
TCGGTTAAGGTGACcCGcATTAAATTGAGCCTTATAG-3’ and 5’-TCATATAAGGTGACCTTATAGG-AGG-3’), URA7allele (primers: 5’-GCA-
TTAGAACTACGcATGTGATTCG-3’ and 5’-CTTGTCATAG-3’), and 5’-CTACGCAGGACACTGAGTTGA-3’).

FIG. 1.

Phosphorylation of Yeast CTP Synthetase

The diagram shows the positions of the glutamine amidase transfer domain, the site involved in CTP product inhibition, and the protein kinase A phosphorylation site in CTP synthetase. The Ser36, Ser330, Ser354, and Ser454 residues within a protein kinase C phosphorylation motif that were mutated to alanine residues are indicated. The numbers on the top of the diagram denote the amino acid positions in the CTP synthetase protein.

The abbreviations used are: PC, phosphatidylcholine; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Constructions of Plasmids—The codons for Ser26, Ser130, Ser354, and Ser454 in the ura7-encoded CTP synthetase were changed to alanine codons by site-directed mutagenesis. The URA7 allele (primers: 5’-CCC-TCGGTTAAGGTGACcCGcATTAAATTGAGCCTTATAG-3’ and 5’-TCATATAAGGTGACCTTATAGG-AGG-3’), URA7 allele (primers: 5’-GCCATGGAACTACGcATGTGATTCG-3’ and 5’-CTTGTCATAG-3’), and 5’-CTACGCAGGACACTGAGTTGA-3’).
Phosphorylation of Yeast CTP Synthetase

**TABLE I**

| Strains and plasmids used in this work |
|---------------------------------------|
| E. coli  |
| DH5α  |
| λ thi-1 gyrA96 recA1 |
| S. cerevisiae  |
| SGY157  |
| MATα leu2-3, 112 trp1-289 ura3-52 |
| SDO195  |
| ura7Δ:TRP1, ura8Δ:hisG derivative of SGY157 bearing URA7-containing plasmids |

**Plasmid**

| pFL44S-URA7  |
| Multicyclic E. coli/yeast shuttle vector containing the URA7 gene and the URA3 marker |
| pDO178  |
| URA7 derivative of pBlueScript II used for mutagenesis |
| pDO105  |
| Multicyclic E. coli/yeast shuttle vector containing the ADH1 promoter and the LEU2 marker |
| YepLac111  |
| Single copy E. coli/yeast shuttle vector containing the LEU2 marker |
| pDO120  |
| Single copy E. coli/yeast shuttle vector containing the ADH11 promoter and the LEU2 marker |
| pTP1  |
| URA7 derivative of pDO105 |
| pTP3  |
| URA7 derivative of pDO120 |
| pTP5  |
| URA7506A derivative of pDO105 |
| pTP6  |
| URA7505A derivative of pDO105 |
| pTP7  |
| URA7505A derivative of pDO120 |
| pTP8  |
| URA7505A derivative of pDO120 |
| pTP9  |
| URA7505A derivative of pDO120 |
| pTP10  |
| URA7505A derivative of pDO120 |
| pTP11  |
| URA7505A derivative of pDO120 |
| pTP12  |
| URA7505A derivative of pDO120 |

**Phosphorylation of CTP Synthetase**—The ATP/UTP-dependent oligomerization of CTP synthetase (5) from the dimeric to the tetrameric form of the enzyme was analyzed by Superose 6 gel filtration chromatography (29). The column was calibrated with blue dextran 2000 (for the void volume), thyroglobulin (669 kDa), apoferitin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and cytochrome c (12.4 kDa). The relative amounts of the dimeric and tetrameric forms of the CTP synthetase in the column fractions were quantified by scanning densitometry of silver-stained SDS-polyacrylamide gels (29).

**Phosphorylation of CTP Synthetase and Synthetic Peptides with Protein Kinase C**—Phosphorylation of CTP synthetase was performed in a total volume of 40 μl at 30 °C. CTP synthetase (1 μg) was incubated for 15 min with 50 mM Tris-HCl (pH 8.0), 50 μM [γ-32P]ATP (4 μCi/μmol), 10 mM MgCl2, 10 mM 2-mercaptoethanol, 0.375 mM EDTA, 0.375 mM EGTA, 1.7 mM CaCl2, 20 μM dicylglycerol, 50 μM phosphatidylserine, and 0.1 nmol/min/mg protein kinase C (35). At the end of the phosphorylation reactions, samples were treated with an equal volume of 2× Laemmli sample buffer (52) followed by SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose paper. The phosphorylated enzyme was visualized by phosphorimaging, and the extent of phosphorylation was quantified using ImageQuant software. Reactions containing synthetic peptides were terminated by spotting an aliquot of the reaction mixture onto phosphocellulose filters. The filters were washed with 75 mM phosphoric acid and then subjected to scintillation counting.

**Phosphorylation of CTP Synthetase and Synthetic Peptides with Protein Kinase C**—Phosphorylation of CTP synthetase was performed in a total volume of 40 μl at 30 °C. CTP synthetase (1 μg) was incubated for 15 min with 50 mM Tris-HCl (pH 8.0), 50 μM [γ-32P]ATP (4 μCi/μmol), 10 mM MgCl2, 10 mM 2-mercaptoethanol, 0.375 mM EDTA, 0.375 mM EGTA, 1.7 mM CaCl2, 20 μM dicylglycerol, 50 μM phosphatidylserine, and 0.1 nmol/min/mg protein kinase C (35). At the end of the phosphorylation reactions, samples were treated with an equal volume of 2× Laemmli sample buffer (52) followed by SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose paper. The phosphorylated enzyme was visualized by phosphorimaging, and the extent of phosphorylation was quantified using ImageQuant software. Reactions containing synthetic peptides were terminated by spotting an aliquot of the reaction mixture onto phosphocellulose filters. The filters were washed with 75 mM phosphoric acid and then subjected to scintillation counting.

**CNBr Digestion and One-dimensional Phosphopeptide Mapping**—The wild type and mutant CTP synthetases were phosphorylated with protein kinase C and γ-32P-ATP for 15 min. The phosphorylated enzymes were subjected to SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose paper. The CTP synthetase on the nitrocellulose paper was isolated and digested with 200 μl of 100 mg/ml CNBr in 70% formic acid for 1.5 h (54). The mixture was centrifuged, and the supernatant was collected and dried in vacuo. The samples were suspended in 0.5 ml of deionized water, dried in vacuo, and then suspended in 20 μl of Laemmli sample buffer (52). Phosphopeptides were separated by SDS-polyacrylamide gel electrophoresis using a 24% slab gel as described by Luo et al. (54). Phosphopeptides were used to identify phosphopeptides in the polyacrylamide gel.

**Enzyme Assays and Protein Determination**—CTP synthetase activity was determined by measuring the conversion of UTP to CTP (molar extinction coefficients of 182 and 1520 M−1 cm−1, respectively) by following the increase in absorbance at 291 nm on a recording spectrophotometer (2). The standard reaction mixture contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 10 mM 2-mercaptoethanol, 2 mM L-glutamine, 0.3 mM GTP, 2 mM ATP, 2 mM UTP, and an appropriate dilution of yeast enzyme protein in a total volume of 0.2 ml. Enzyme assays were performed in triplicate with an average S.D. of ±3%. All assays were linear with time and protein concentration. A unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product/minute. Protein concentration was estimated...
by the method of Bradford (55) using bovine serum albumin as the standard.

Analysis of CTP—Cells expressing the wild type and the S36A, S330A, S354A, and S454A mutant CTP synthetases from single copy plasmids were grown to the exponential phase of growth. CTP was extracted (6), and its concentration was analyzed by high performance liquid chromatography as described by Fappas et al. (56).

Analysis of Phosphoproteins—Phospholipids were labeled with [32P] and [methyl-3H]choline as described previously (17, 57, 58). Phospholipids were extracted from labeled cells by the method of Bligh and Dyer (59) as described previously (60). Phospholipids were analyzed by two-dimensional thin-layer chromatography on silica gel thin-layer chromatography plates. The solvent systems for dimensions one and two were chloroform, methanol, glacial acetic acid (65:25:10, v/v/v) and chloroform, methanol, 8% formic acid (65:25:10, v/v/v), respectively (61). The radio-labeled phospholipids were visualized by phosphorimaging analysis. The positions of the labeled lipids on chromatography plates were compared with standard phospholipids after exposure to iodine vapor. The amount of each labeled phospholipid was determined by liquid scintillation counting.

Analyses of Data—Kinetic data were analyzed with the EZ-FIT enzyme kinetic model-fitting program (62). Statistical analyses were performed with SigmaPlot software.

RESULTS

CTP Synthetase Synthetic Peptides Containing a Protein Kinase C Sequence Motif Are Substrates for Protein Kinase C—Analysis of the deduced sequence of CTP synthetase revealed that the protein has potential serine (Ser<sup>36</sup>, Ser<sup>330</sup>, Ser<sup>354</sup>, and Ser<sup>454</sup>) and threonine (Thr<sup>90</sup>, Thr<sup>303</sup>, Thr<sup>454</sup>, and Thr<sup>529</sup>) phosphorylation sites within a protein kinase C sequence motif. Peptides containing the serine (LKVTS36IKIDP, LEHSS330MKCRR, EAQES354NKTKF, and TMGG544MRGLL) and threonine (HNIT90GKIYS, ESMET303VKIRL, GKDDT514GRIE, and HPEYT539SKVLD) target sites, respectively, were synthesized based on the protein sequence of CTP synthetase. The four peptides containing sequences for the potential serine phosphorylation sites served as substrates for protein kinase C in a concentration-dependent manner (Fig. 2). Of the four peptides, the Ser<sup>330</sup> peptide was the best substrate for protein kinase C (data not shown).

Construction and Characterization of CTP Synthetase S36A, S330A, S354A, and S454A Mutants—Mutagenesis of Ser<sup>36</sup>, Ser<sup>330</sup>, Ser<sup>354</sup>, and Ser<sup>454</sup> within the CTP synthetase was performed to further examine the hypothesis that these sites might be targets for protein kinase C. CTP synthetases with serine to alanine (S36A, S330A, S354A, and S454A) mutations were constructed by site-directed mutagenesis and expressed on single copy and multicopy plasmids in the ura7<sup>A</sup> ara8<sup>A</sup> double mutant. Cells bearing the wild type and mutant alleles of the URA7 gene exhibited similar growth rates when grown vegetatively at 30 °C in minimal synthetic media and in rich YEPD. No morphological differences were observed in cells bearing the mutant enzymes. Antibodies directed against the wild type CTP synthetase (63) recognized the mutant enzymes (Fig. 3A). Scanning densitometry of the immunoblot shown in Fig. 3A showed that there were no major differences in the expression of the wild type and mutant CTP synthetases in cells bearing the single copy plasmids. Thus, the S36A, S330A, S354A, and S454A mutations in the URA7 gene did not affect the functional expression of the enzyme.

Cells expressing the wild type and mutant CTP synthetase alleles on the single-copy plasmid were labeled with [32P] to detect phosphorylated CTP synthetase and with U-<sup>14</sup>C-labeled L-α-amino acids to normalize for the amount of CTP synthetase isolated. The ratio of counts/min of [32P] incorporated into CTP synthetase to the counts of [14C] incorporated into CTP synthetase was used to examine the extent of phosphorylation in vivo (35). The wild type and mutant CTP synthetases were isolated by immunoprecipitation, and the amount of each label incorporated into the enzymes was determined. The phosphorylation state of the S36A, S330A, and S454A mutant enzymes was reduced by 37, 37, and 30%, respectively, when compared with the wild type control enzyme (Fig. 3B). On the other hand, the S330A mutation did not have a significant effect on the phosphorylation state of CTP synthetase (Fig. 3B).

We next examined the levels of CTP synthetase activity in cells bearing the mutations. The specific activity in cells with the S36A, S330A, and S454A mutations was reduced by 53, 63, and 10%, respectively, when compared with cells bearing the wild type enzyme (Fig. 3C). In contrast, the CTP synthetase activity in cells bearing the S330A mutant enzyme was elevated by 64% (Fig. 3C). CTP synthetase specific activity was based on the total protein concentration in cell extracts.

Partial Purification of the S36A, S330A, S354A, and S454A Mutant CTP Synthetases—The wild type and the S36A, S330A, S354A, and S454A mutant CTP synthetase enzymes were purified to examine the effects of the mutations on the properties of the enzyme. The purification scheme developed by Yang et al. (5) was modified to obtain partially purified preparations of the enzyme in a relatively short amount of time. A 50-fold overexpression of the URA7 gene on the multicopy plasmid facilitated the purification of the enzymes. The purification scheme included ammonium sulfate fractionation of the cytosol.
followed by chromatography with Superose 6 and with Mono Q. The Superose 6 chromatography step purified the enzyme and served as a convenient desalting step. The Mono Q chromatography step purified the enzyme and at the same time was effective in concentrating the enzyme. The mutant enzymes behaved similarly to the wild type enzyme during each step of the purification. Analysis by SDS-polyacrylamide gel electrophoresis showed that the purification scheme resulted in highly purified preparations of the wild type and mutant CTP synthetases (Fig. 4). The yield (17–27%) and degree of purification (20–26-fold) of the mutant enzymes were similar to that of the wild type control enzyme (Table II). The specific activity of the partially purified enzyme preparations ranged from 0.66 to 0.2 unit/mg. For reference, the specific activity of pure wild type CTP synthetase is typically 2.3–2.5 units/mg (5, 36).

**Effects of the S36A, S330A, S354A, and S454A Mutations on the Phosphorylation of CTP Synthetase by Protein Kinase C in Vitro**—The effects of the S36A, S330A, S354A, and S454A mutations on the phosphorylation of CTP synthetase by protein kinase C were examined. The SDS gel-purified CTP synthetase proteins were used for these experiments. In control experiments, we showed that this procedure did not affect the phosphorylation of the enzyme by protein kinase C. Samples of the wild type and mutant enzymes were incubated with protein kinase C and 32P-labeled ATP. After the phosphorylation reactions, samples were subjected to SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose paper and phosphorimaging analysis. The phosphorylation of CTP synthetase by protein kinase C was most affected by the S330A mutation. The S330A mutation caused a 70% decrease for label incorporation into the enzyme (Fig. 5A). The S36A, S354A, and S454A mutations did not have a great effect on the ability of protein kinase C to phosphorylate CTP synthetase in vitro.

These results were consistent with the observation that protein kinase C activity was 100-fold greater using the synthetic peptide containing the phosphorylation motif at Ser330 when compared with the other peptide substrates.

The phosphorylated wild type and mutant CTP synthetase enzymes were subjected to CNBr cleavage and one-dimensional phosphopeptide mapping analysis (Fig. 5B). Six phosphopeptides (labeled a through f) were derived from the wild type and mutant enzymes. The most heavily phosphorylated peptides

**FIG. 3. Effects of the S36A, S330A, S354A, and S454A Mutations on the expression, the state of phosphorylation, and activity of CTP synthetase.** Cells expressing wild type (WT) and the indicated S36A, S330A, S354A, and S454A mutant CTP synthetase enzymes from the single-copy plasmids were grown to the exponential phase of growth. **Panel A**, cell extracts were prepared and subjected to immunoblot analysis using anti-CTP synthetase antibodies. A portion of the immunoblot shows the expression of wild type and mutant CTP synthetase enzymes. Relative amounts of CTP synthetase protein were determined by densitometry scanning. The amount of CTP synthetase protein found in cells bearing the wild type enzyme was set as 1. The position of CTP synthetase is indicated. **Panel B**, cells were labeled with 32Pi and U-14C-labeled L-amino acids. The CTP synthetase proteins were immunoprecipitated from cell extracts using anti-CTP synthetase antibodies. CTP synthetase was dissociated from the enzyme-antibody complex, and the amount of the label incorporated into CTP synthetase was determined by scintillation counting. The values are reported as the cpm of 32P incorporated into CTP synthetase relative to the cpm of 14C incorporated into CTP synthetase. The values reported were the averages of three separate experiments ± S.D. **Panel C**, cells extracts were prepared and assayed for CTP synthetase activity. The specific activity (µmol/min/mg) was determined using the total protein concentration in cell extracts. The values reported were the averages of three separate experiments ± S.D.

**FIG. 4. SDS-polyacrylamide gel electrophoresis of purified wild type and S36A, S330A, S354A, and S454A mutant CTP synthetases.** Partially purified wild type (WT) and the indicated S36A, S330A, S354A, and S454A mutant CTP synthetases were subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. The protein molecular mass standards (Std) from top to bottom are phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa). The position of CTP synthetase is indicated in the figure.

**TABLE II**  
| CTP synthetase | Specific activity | Yield | Purification |
|----------------|------------------|-------|-------------|
| Wild type      | 0.66             | 23    | 26.0        |
| S36A           | 0.30             | 17    | 25.4        |
| S330A          | 0.83             | 22    | 20.3        |
| S354A          | 0.20             | 21    | 25.9        |
| S454A          | 0.60             | 27    | 25.7        |

**Effects of the S36A, S330A, S354A, and S454A Mutations on the Phosphorylation of CTP Synthetase by Protein Kinase C in Vitro**—The effects of the S36A, S330A, S354A, and S454A mutations on the phosphorylation of CTP synthetase by protein kinase C were examined. The SDS gel-purified CTP synthetase proteins were used for these experiments. In control experiments, we showed that this procedure did not affect the phosphorylation of the enzyme by protein kinase C. Samples of the wild type and mutant enzymes were incubated with protein kinase C and 32P-labeled ATP. After the phosphorylation reactions, samples were subjected to SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose paper and phosphorimaging analysis. The phosphorylation of CTP synthetase by protein kinase C was most affected by the S330A mutation. The S330A mutation caused a 70% decrease for label incorporation into the enzyme (Fig. 5A). The S36A, S354A, and S454A mutations did not have a great effect on the ability of protein kinase C to phosphorylate CTP synthetase in vitro. These results were consistent with the observation that protein kinase C activity was 100-fold greater using the synthetic peptide containing the phosphorylation motif at Ser330 when compared with the other peptide substrates.

The phosphorylated wild type and mutant CTP synthetase enzymes were subjected to CNBr cleavage and one-dimensional phosphopeptide mapping analysis (Fig. 5B). Six phosphopeptides (labeled a through f) were derived from the wild type and mutant enzymes. The most heavily phosphorylated peptides
bated with protein kinase C and 32P-labeled ATP for 15 min. After incubation, samples were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose paper. The phosphorylated proteins were subjected to phosphorimaging analysis, and the relative amounts of phosphate incorporated were quantified using ImageQuant software. The relative phosphorylation of the wild type CTP synthetase protein was set as 1. The position of CTP synthetase is indicated in the figure. Panel B, the labeled proteins were excised from the nitrocellulose paper and subjected to CNBr cleavage. The resulting CNBr cleavage products, Ser330 should be contained in phosphopeptide c whereas Ser36 should be contained in phosphopeptide d. The amount of phosphopeptide c was also elevated in the S330A mutant enzyme. Based on the predicted sizes of the CNBr cleavage products, Ser330 should be contained in phosphopeptide c and d. Of the four mutations, the S330A mutation had the greatest effect on the phosphopeptide map of CTP synthetase. The major effects of the S330A mutation were decreases in the amounts of phosphopeptides c and d and an increase for phosphopeptide f. The amount of phosphopeptide f was also elevated in the S354A mutant enzyme. Based on the predicted sizes of the CNBr cleavage products, Ser330 should be contained in phosphopeptide d.

Effects of the S36A, S330A, S354A, and S454A Mutations on the Enzymic Properties of CTP Synthetase—A kinetic analysis was performed to further characterize the effects of the S36A, S330A, S354A, and S454A mutations on CTP synthetase activity. Equal amounts (as determined by densitometry scanning of SDS-polyacrylamide gels) of the wild type and mutant proteins were used for these experiments. The dependence of CTP synthetase activity on UTP (Fig. 6A) and ATP (Fig. 6B) was examined using a subsaturating concentration of UTP and ATP, respectively. Under these conditions, we could more readily observe stimulatory or inhibitory effects of the mutations on enzyme activity. The S36A and S354A mutations caused a decrease in CTP synthetase activity when measured with respect to UTP and with respect to ATP. Conversely, the S330A mutation caused an increase in activity when measured with respect to each substrate. The S36A and S354A mutations caused the inhibition of CTP synthetase activity by a mechanism that primarily affected the apparent $V_{\text{max}}$ values with respect to UTP and ATP (Table III). The S330A mutation caused the stimulation of activity by a mechanism that primarily affected the apparent $V_{\text{max}}$ with respect to UTP and ATP (Table III). The S454A mutation did not have a significant effect on CTP synthetase activity except for a small decrease in the apparent $K_{\text{m}}$ value for ATP (Table III). The mutations did not affect the Hill number ($n = 1.4$) for UTP.

The activity of the wild type CTP synthetase enzyme is inhibited by CTP (5). Because protein kinase C phosphorylation of pure wild type CTP synthetase results in a decrease in the enzyme sensitivity to product inhibition by CTP (35), we examined the effects of the mutations on this property. As described previously (5), the $IC_{50}$ for CTP inhibition of wild type CTP synthetase activity was 0.3 mM. None of the mutations had a significant effect on this property. This suggests that the role of protein kinase C phosphorylation on this enzyme property may be more complicated than the phosphorylation of any one site. We also examined the effects of the S36A, S330A, S354A, and S454A mutations on the pH optimum of the CTP synthetase reaction. As described previously (5), the pH optimum for wild type CTP synthetase activity was 8.0, and the mutations did not have an effect on this property.

Effects of the S36A, S330A, S354A, and S454A Mutations on the Oligomerization of CTP Synthetase—In vitro, wild type CTP synthetase exists as an inactive dimer that oligomerizes to an active tetramer in the presence of its substrates ATP and UTP (5). Data indicate that this process is facilitated by protein interaction between the active site and regulatory domains of the tetrameric enzyme. The S330A mutation had the greatest effect on CTP synthetase oligomerization. The S36A, S354A, and S454A mutations caused the stimulation of activity by a mechanism that primarily affected the apparent $V_{\text{max}}$ values with respect to UTP and ATP (Table III). The S330A mutation caused the stimulation of activity by a mechanism that primarily affected the apparent $V_{\text{max}}$ with respect to UTP and ATP (Table III). The S454A mutation did not have a significant effect on CTP synthetase activity except for a small decrease in the apparent $K_{\text{m}}$ value for ATP (Table III). The mutations did not affect the Hill number ($n = 1.4$) for UTP.
kinase C phosphorylation of the enzyme (29). Accordingly, we questioned whether the S36A, S330A, S354A, and S454A mutations in CTP synthetase affected this property of the enzyme. The purified preparations of the wild type and mutant enzymes were subjected to Superose 6 chromatography in the absence and presence of subsaturating concentrations of ATP and UTP. Under these conditions, we could more readily observe the effects of the mutations on the oligomerization of CTP synthetase. The amount of the inactive dimeric form of the S36A and S354A mutant CTP synthetase enzymes was 54 and 98%, respectively, when compared with the wild type enzyme (Fig. 7). The S330A and S454A mutations did not have a major effect on the oligomerization of CTP synthetase.

Effects of the S36A, S330A, S354A, and S454A Mutations in CTP Synthetase on the Cellular Concentration of CTP—We questioned whether the changes in CTP synthetase activity that were brought about by the S36A, S330A, S354A, and S454A mutations would affect the levels of CTP in vivo. Cells bearing the wild type and mutant CTP synthetases expressed from the single-copy plasmid were grown to the exponential phase of growth, and CTP was extracted and analyzed by high performance liquid chromatography. The concentration of CTP in cells with the S36A, S354A, and S454A mutant enzymes was reduced by 40, 50, and 11%, respectively, whereas the concentration of CTP in cells with the S330A mutant enzyme was elevated by 38% (Fig. 8).

Effects of the S36A, S330A, S354A, and S454A Mutations in CTP Synthetase on Phospholipid Composition—CTP is essential for the synthesis of PC, the major membrane phospholipid in S. cerevisiae (64–67). There are two pathways by which PC is synthesized from CTP. In one pathway, PC is synthesized from CTP via CDP-choline, whereas in the other pathway PC is synthesized from CTP via CDP-diacylglycerol (64–67). The synthesis of PC from CDP-choline is direct. The synthesis of PC from CDP-diacylglycerol is indirect and occurs by the reaction sequence CDP-diacylglycerol → phosphatidylethanolamine → phosphatidylcholine. We questioned whether the S36A, S330A, S354A, and S454A mutations in CTP synthetase would affect phospholipid composition. Cells bearing the wild type and mutant enzymes expressed from the single-copy plasmids were grown to the exponential phase of growth, and nucleotides were extracted, and the concentration of CTP in cells with the S330A mutation had the opposite effect.

Phosphorylation of Yeast CTP Synthetase

**TABLE III**

| CTP synthetase | UTP | ATP |
|----------------|-----|-----|
|                | $V_{max}$ (app) | $K_m$ (app) | $V_{max}$ (app) | $K_m$ (app) |
| Wild type      | 0.37 | 0.11 | 0.28 | 0.45 |
| S36A           | 0.35 | 0.11 | 0.28 | 0.26 |
| S330A          | 0.12 | 0.18 | 0.16 | 0.44 |
| S354A          | 0.10 | 0.07 | 0.11 | 0.36 |
| S454A          | 0.88 | 0.10 | 0.68 | 0.57 |

* Kinetic constants determined with 0.5 mM ATP.
* Kinetic constants determined with 0.1 mM UTP.
The incorporation of 32Pi and experiments/H11006 generations with 32Pi (5 in CTP synthetase on PC synthesis. Cells expressing the wild type were reported as the cpm of 3H incorporated into PC relative to the cpm analyzed by two-dimensional thin-layer chromatography. The data for protein kinase C. Based on this information and previous served as substrates for protein kinase C. This assay provided vivo (33), we constructed Ser S454A) mutations in CTP synthetase to further examine the hypothesis that these sites were targets of protein kinase C. The mutant enzymes were functional and prokaryotic organisms (18). Regulation of this enzyme is plays a major role in the growth and metabolism of eukaryotic and prokaryotic organisms (18). One of the mechanisms by which CTP synthetase activity is regulated in S. cerevisiae is by phosphorylation via protein kinase C (33, 35). Protein kinase C is a lipid-dependent protein kinase required for S. cerevisiae cell cycle (74–78) and plays a role maintaining cell wall integrity (79). In mammalian cells, protein kinase C plays a central role in the transduction of lipid second messengers generated by receptor-mediated hydrolysis of membrane phospholipids (80–82). Phosphorylation of CTP synthetase by protein kinase C in yeast may represent a mechanism by which lipid signaling transduction pathways are coordinately regulated to CTP synthesis and cell growth. Identification of the protein kinase C phosphorylation sites in CTP synthetase is necessary for gaining information about the physiological significance of this phosphorylation. Peptides with sequences for potential serine and threonine protein kinase C phosphorylation sites in CTP synthetase were synthesized and examined for their ability to serve as substrates for protein kinase C in vitro. The peptides containing a phosphorylation motif at Ser36, Ser330, Ser354, and Ser454 served as substrates for protein kinase C. This assay provided evidence that these residues might be phosphorylation sites for protein kinase C. Based on this information and previous data indicating that only serine residues are phosphorylated in vitro (33), we constructed Ser → Ala (S36A, S330A, S354A, and S454A) mutations in CTP synthetase to further examine the hypothesis that these sites were targets of protein kinase C. The mutant enzymes were functional in vitro as evidenced by the suppression of the lethal phenotype (7) of the ura7 ura8 double mutant, and immunoblot analysis showed that the phosphorylation site mutations did not affect the expression of CTP synthetase. Moreover, the mutations did not have a major effect on the overall structure of the CTP synthetase protein. The mutant enzymes behaved normally during purification and exhibited the property of nucleotide-dependent tetramerization.

Consistent with the loss of a phosphorylation site, the phosphorylation state of CTP synthetase in vivo was reduced in cells carrying the S36A, S354A, and S454A mutations. On the other hand, the phosphorylation state of the S330A mutant enzyme was not altered in vivo. An explanation for this result is that the loss of Ser330 as a phosphorylation site resulted in the phosphorylation of the enzyme at another site. This phosphorylation could be due to protein kinase C or another protein kinase. That protein kinase C may be involved in vivo was supported by the in vitro phosphorylation experiment where the S330A mutation caused a decrease in phosphopeptides c and d and an increase in phosphopeptide f.

The CTP synthetase activity in extracts from cells bearing the S36A, S354A, and S454A mutant enzymes was reduced when compared with cells bearing the wild type enzyme. In addition, the kinetic analysis of the purified mutant enzymes revealed that the S36A and S354A mutations caused a decrease in the Vmax of the reaction. These results were consistent with previous data showing that protein kinase C phosphorylation stimulates the activity of the pure wild type CTP synthetase (33, 35). Interestingly, the CTP synthetase activity in cells bearing the S330A mutant enzyme was elevated, and kinetic analysis of the purified enzyme showed that the S330A mutation caused an elevation in the Vmax of the reaction. These data raised the suggestion that the phosphorylation of Ser330 causes the inhibition of CTP synthetase activity. Thus, the effect of phosphorylation on CTP synthetase activity in vivo might be governed by which site(s) is phosphorylated.

Previous data using the purified wild type CTP synthetase indicated that protein kinase C phosphorylation facilitates the ATP/UTP-dependent oligomerization of the enzyme (29). Of the four mutations, only the S36A and the S354A mutations affected the dimer to tetramer conversion of CTP synthetase. These mutations caused a greater amount of the enzyme to exist in its inactive dimeric form when compared with the wild type control enzyme. Thus, the lower activity exhibited by the S36A and S354A mutant enzymes may be attributed to the effect of phosphorylation of Ser36 and Ser354 on the nucleotide-dependent oligomerization of CTP synthetase. This analysis indicated that phosphorylation of Ser330 and Ser454 did not have a major effect on the oligomerization of the enzyme.

We initiated studies to examine the physiological consequences of the S36A, S330A, S354A, and S454A mutations in CTP synthetase. The concentration of CTP in cells bearing the S36A, S354A, and S454A mutant enzymes was reduced by 40, 50, and 11%, respectively, whereas the CTP concentration in cells bearing the S330A mutation was elevated by 58%. These data were consistent with the effects of the mutations on CTP synthetase activity and provided further support that Ser36, Ser330, Ser354, and Ser454 are phosphorylation sites in CTP synthetase. Previous studies show that the cellular levels of CTP affect the pathways by which PC is synthesized (17, 30). For example, PC synthesis via the CDP-choline pathway is stimulated when CTP levels are elevated due to the misregulation of CTP synthetase activity by CTP product inhibition (30). Given the fact that the cellular levels of CTP were altered in cells bearing the S36A, S330A, S354A, and S454A mutations, we examined the effects of the mutations on phospholipid composition. The in vivo labeling experiments showed that the amount of PC synthesized via the CDP-choline pathway was reduced in cells with the S36A, S354A, and S454A mutations, whereas the amount of PC synthesized via this pathway was elevated in cells with the S330A mutation. The mechanism for this regulation may be attributed to the availability of CTP for phosphocholine cytidylyltransferase (17), the rate-limiting enzyme in the CDP-choline pathway (67, 73, 83). The cellular concentrations of CTP in cells with the wild type and the S36A,
Phosphorylation of Yeast CTP Synthetase

S330A, S354A, and S454A mutant CTP synthetase enzymes were within the range of the $K_m$ value (1.4 mM) of CTP for the phosphocholine cytidylyltransferase (84). Thus, phosphocholine cytidylyltransferase activity would be sensitive to the changes in CTP levels brought about by the mutations in CTP synthetase. The CDP-diacylglycerol synthase enzyme may also be sensitive to the cellular concentrations of CTP because its $K_m$ value for CTP is 1 mM (85). However, in contrast to the phosphocholine cytidylyltransferase reaction, the synthesis of CDP-diacylglycerol is not a rate-limiting step in the CDP-diacylglycerol pathway for PC synthesis (86). Moreover, the synthesis of CDP-diacylglycerol is several steps upstream in the CDP-diacylglycerol-dependent pathway for the synthesis of PC (64–67).

Several proteins are phosphorylated on multiple sites by one or more protein kinases (87–92). For example, the phosphorylation of one site can affect the phosphorylation of another site (i.e., hierarchical phosphorylation (87)). Data indicated that phosphorylation of CTP synthetase at one site affected the phosphorylation of the enzyme at another site. Moreover, the phosphorylation of CTP synthetase at different sites had opposite effects on enzyme activity. CTP synthetase is phosphorylated by protein kinase A, and it is unknown whether the phosphorylation of CTP synthetase by protein kinase A and vice versa. Moreover, data suggest that CTP synthetase is phosphorylated by yet additional protein kinases (34). Thus, the regulation of CTP synthetase by phosphorylation is very complex. In this work, we addressed some of this complex regulation by identifying sites that are phosphorylated by protein kinase C and show that this phosphorylation is physiologically relevant.

Acknowledgments—We acknowledge Gis-Soo Han, Darin Ostrander, Avula Sreenivas, and David Toke for helpful discussions and assistance during the course of this work.

REFERENCES

1. Liberman, I. (1956) J. Biol. Chem. 225, 765–775
2. Long, C. W., and Purcell, A. B. (1967) J. Biol. Chem. 242, 4715–4721
3. Levitzki, A., and Koshland, D. E., Jr. (1972) Biochemistry 11, 241–246
4. Bearne, S. L., Hekmat, O., and Macdonnell, J. E. (2001) Biochem. J. 356, 225–232
5. Yang, W.-L., McKinney, V. M., Ozier-Kalogeropoulos, O., Adeline, M.-T., Collin, J., and Lacroute, F. (1994) EMBO J. 13, 71–76
6. Ozier-Kalogeropoulos, O., Adeline, M.-T., Yang, W.-L., Carman, G. M., and Lacroute, F. (1994) Mol. Gen. Genet. 242, 431–439
7. Yang, W.-L., and Carman, G. M. (1995) J. Biol. Chem. 270, 18774–18780
8. Han, D. L., Yang, W.-L., and Carman, G. M. (1996) J. Biol. Chem. 271, 11113–11119
9. Barbas, T. S., Ostrander, D. B., Pappas, A., and Carman, G. M. (1999) Biochemistry 38, 8839–8848
10. Tipples, G., and McClarty, G. (1995) J. Biol. Chem. 270, 18992–18997
11. Choeck, Ph., Bhee, S. G., and Stadtman, E. R. (1980) Adv. Enzyme Regul. 18, 765–779
12. Watanabe, M., Chen, C.-Y., and Levin, D. E. (1994) J. Biol. Chem. 269, 16829–16836
13. Laemmli, U. K. (1970) Nature 227, 680–685
14. Haid, A., and Sussma, M. (1983) Methods Enzymol. 96, 192–205
15. Luo, K. X., Hurley, T. R., and Sefton, B. M. (1991) Methods Enzymol. 201, 149–153
16. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
17. Pappas, A., Park, T.-S., and Carman, G. M. (1999) Biochemistry 38, 16671–16677
18. Adonap, K., Fugel, S., and Henry, S. A. (1980) J. Biol. Chem. 255, 6651–6661
19. Homann, M. J., Poole, M. A., Gaynor, P. M., Ho, C.-T., and Carman, G. M. (1987) J. Biol. Chem. 262, 533–539
20. Eister, G. E., and Dyer, W. E. (2000) J. Biol. Chem. 275, 9111–9117
21. Morlock, K. R., Lin, Y.-P., and Carman, G. M. (1988) J. Biol. Chem. 263, 3561–3566
22. Brizzol, J. B., and Bixler, C. H. (1980) J. Biol. Chem. 255, 4474–4480
23. Perrella, F. (1988) Anal. Biochem. 174, 437–447
24. Wu, W.-L., Liu, Y., Riedel, B., Wissing, J. B., Fischl, A. S., and Carman, G. M. (1996) J. Biol. Chem. 271, 1868–1875
25. Palcauf, F., Kohlibre, S. D., and Henry, S. A. (1992) in The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression (Jones, E. W., Pringle, J. R., and Brach, J. R., eds) pp. 415–500, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Revier, D., O'Brien, D. J., Gorman, J. A., and Carman, G. M. (1998) J. Biol. Chem. 273, 18992–19001
27. Choeck, Ph., Bhee, S. G., and Stadtman, E. R. (1980) Adv. Enzyme Regul. 18, 765–779
28. Weber, G., Olah, E., Lui, M. S., and Teng, D. (1979) Adv. Enzyme Regul. 17, 1–21
29. Verschuer, A. C., van Gennip, A. H., Brinkman, J., Voute, P. A., and Van Kuilenburg, A. B. (2000) Adv. Exp. Med. Biol. 486, 315–325
30. Verschuer, A. C., Brinkman, J., von Gennip, A. H., Leen, R., Vet, J. R., Evers, L. M., Voute, P. A., and Van Kuilenburg, A. B. (2001) Leuk. Res. 25, 891–900
31. Schafani, R. A., and Fangman, W. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5201–5206
32. Nakdarni, A. K., McDonough, V. M., Yang, W.-L., Stueky, J. E., Ozier-Kalogeropoulos, O., and Carman, G. M. (1995) J. Biol. Chem. 270, 24982–24988
33. Pappas, A., Yang, W.-L., Park, T.-S., and Carman, G. M. (1998) J. Biol. Chem. 273, 15054–15060
34. Ostrander, D. B., O'Brien, D. J., Gorman, J. A., and Carman, G. M. (1998) J. Biol. Chem. 273, 18992–19001
35. Culbertson, M. R., and Henry, S. A. (1975) J. Biol. Chem. 250, 5292–5297
36. Baumber, M. R., and Brinkman, J., and Voute, P. A. (1980) Adv. Exp. Med. Biol. 111, 241–245
37. Schiestl, R. H., and Gietz, D. (1989) Curr. Biol. 16, 339–346
38. Haid, A., and Sussma, M. (1983) Methods Enzymol. 96, 192–205
39. Fouks, R. S., and Boeke, J. D. (1991) Methods Enzymol. 194, 302–318
40. Warner, J. R. (1991) Methods Enzymol. 194, 425–428
41. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
42. Culbertson, M. R., and Henry, S. A. (1975) J. Biol. Chem. 250, 5292–5297
83. Vance, D. E. (1991) in *Biochemistry of Lipids, Lipoproteins, and Membranes* (Vance, D. E., and Vance, J., eds) pp. 205–240, Elsevier Science Publishers B. V., Amsterdam
84. Nikawa, J., Yonemura, K., and Yamashita, S. (1983) *Eur. J. Biochem.* **131**, 223–229
85. Kelley, M. J., and Carman, G. M. (1987) *J. Biol. Chem.* **262**, 14563–14570
86. Kelley, M. J.; Bailis, A. M.; Henry, S. A., and Carman, G. M. (1988) *J. Biol. Chem.* **263**, 18078–18085
87. Roach, R. J. (1991) *J. Biol. Chem.* **266**, 14139–14142
88. Roach, P. J. (1990) *FASEB J.* **4**, 2961–2968
89. Lutterbach, B., and Hann, S. R. (1994) *Mol. Cell. Biol.* **14**, 5510–5522
90. Jicha, G. A., O’Donnell, A., Weaver, C., Angeletti, R., and Davies, P. (1999) *J. Neurochem.* **72**, 214–224
91. Maestri-El Kouhen, O. F., Wang, G., Solberg, J., Erickson, L. J., Law, P. Y., and Loh, H. H. (2000) *J. Biol. Chem.* **275**, 36659–36664
92. Vida, T. A., and Emr, S. D. (1995) *J. Cell Biol.* **128**, 779–792

**Phosphorylation of Yeast CTP Synthetase**