Identification of Ser^{424} as the Protein Kinase A Phosphorylation Site in CTP Synthetase from *Saccharomyces cerevisiae*[†]

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ABSTRACT: The *URA7*-encoded CTP synthetase [EC 6.3.4.2, UTP:ammonia ligase (ADP-forming)] in the yeast *Saccharomyces cerevisiae* is phosphorylated on a serine residue and stimulated by cAMP-dependent protein kinase (protein kinase A) in vitro. In vivo, the phosphorylation of CTP synthetase is mediated by the *RAS*/cAMP pathway. In this work, we examined the hypothesis that amino acid residue Ser^{424} contained in a protein kinase A sequence motif in the *URA7*-encoded CTP synthetase is the target site for protein kinase A. A CTP synthetase synthetic peptide (SLG**RKDS**HSA) containing the protein kinase A motif was a substrate (K_m = 30 μM) for protein kinase A. This peptide also inhibited (IC_{50} = 45 μM) the phosphorylation of purified wild-type CTP synthetase by protein kinase A. CTP synthetase with a Ser^{424} → Ala (S424A) mutation was constructed by site-directed mutagenesis. The mutated enzyme was not phosphorylated in response to the activation of protein kinase A activity in vivo. Purified S424A mutant CTP synthetase was not phosphorylated and stimulated by protein kinase A. The S424A mutant CTP synthetase had reduced V_{max} and elevated K_m values for ATP and UTP when compared with the protein kinase A-phosphorylated wild-type enzyme. The specificity constants for ATP and UTP for the S424A mutant CTP synthetase were 4.2- and 2.9-fold lower, respectively, when compared with that of the phosphorylated enzyme. In addition, the S424A mutant enzyme was 2.7-fold more sensitive to CTP product inhibition when compared with the phosphorylated wild-type enzyme. These data indicated that the protein kinase A target site in CTP synthetase was Ser^{424} and that the phosphorylation of this site played a role in the regulation of CTP synthetase activity.

In the yeast *Saccharomyces cerevisiae*, CTP synthetase (EC 6.3.4.2) catalyzes the final step in the pyrimidine biosynthetic pathway (*1*). The enzyme catalyzes the ATP-dependent transfer of the amide nitrogen from glutamine to the C-4 position of UTP to form CTP. GTP is an allosteric effector which accelerates the formation of a covalent glutaminyl enzyme catalytic intermediate (*2*−*4*). Two duplicate genes in *S. cerevisiae* named *URA7* (*1*) and *URA8* (*5*) encode CTP synthetase. The deduced protein products of the *URA7* and *URA8* genes contain a conserved glutamine amide transfer domain common to CTP synthetases from other organisms (*6*−*9*). The *URA7*-encoded CTP synthetase is more abundant than the *URA8*-encoded enzyme (*10*) and is responsible for the majority of the CTP synthesized in vivo (*5*). Neither the *URA7* nor the *URA8* gene is essential as long as cells possess one functional CTP synthetase gene (*1*, *5*). The essential nature of CTP synthetase emanates from the fact that the reaction product CTP is required for the synthesis of RNA, DNA, membrane phospholipids, and sialoglycoproteins (*11*).

The *URA7* (*4*)- and *URA8*-encoded (*12*) CTP synthetases have been purified to homogeneity from the cytosolic fraction of the cell and characterized with respect to their enzymological and kinetic properties. These CTP synthetases exhibit positive cooperative kinetics with respect to UTP and ATP (*4*, *12*). Studies with the *URA7*-encoded CTP synthetase indicate that the cooperative kinetics of the enzyme are due to the nucleotide-dependent oligomerization of an inactive dimeric form to an active tetrameric form of the enzyme (*13*).

The *URA7* (*4*)- and *URA8*-encoded (*12*) CTP synthetases are regulated by CTP product inhibition. The inhibition of CTP synthetase activity by CTP regulates the cellular concentration of CTP in growing cells (*4*, *10*, *14*). An E161K mutation in the *URA7*-encoded CTP synthetase renders the enzyme defective in the regulation of activity by CTP product inhibition (*14*). Cells carrying this mutant enzyme exhibit elevated levels of CTP and alterations in the regulation of phospholipid metabolism (*14*). The major consequence of the mutation on phospholipid metabolism is an increase in phosphatidylcholine content and an increase the level of utilization of the CDP-choline pathway for phosphatidylcholine synthesis (*14*).

The *URA7*-encoded CTP synthetase is also regulated by phosphorylation via protein kinases A (*15*) and C (*16*, *17*). In vitro, phosphorylation of the *URA7*-encoded CTP synthetase by protein kinases A (*15*) and C (*16*, *17*) results in

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Table 1: Strains and Plasmids Used in This Work

strain or plasmid	relevant characteristics	ref
E. coli		
DH5α	F$^-$, ϕ80d*lac*ZΔM15, Δ(*lacZYA-arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(r_k^- m_k^+), *pho*A, *sup*E44, λ^- *thi*-1, *gyr*A96, *rel*A1	*19*
S. cerevisiae		
OK8	*MAT*α, *leu2, trp1, ura3, ura7*Δ::*TRP1, ura8*	*5*
plasmid		
pFL44S-*URA7*	2 μm-based multicopy yeast shuttle vector containing the *URA7* gene and the *URA3* marker	*1*
pDO178	*URA7* derivative of pBlueScript II used for mutagenesis	*14*
pDO105	2 μm-based multicopy yeast shuttle vector containing the *ADH1* promoter and the *LEU2* marker	*14*
YepLac111	Cen-based single-copy yeast shuttle vector containing the *LEU2* marker	*24*
pDO120	Cen-based single-copy yeast shuttle vector containing the *ADH1* promoter and the *LEU2* marker	this study
pTP1	*URA7* derivative of pDO105	this study
pTP2	*URA7*^{S424A} derivative of pDO105	this study
pTP3	*URA7* derivative of pDO120	this study
pTP4	*URA7*^{S424A} derivative of pDO120	this study

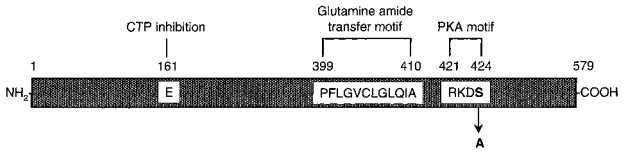

FIGURE 1: Domain structure of the *URA7*-encoded CTP synthetase. The diagram shows the positions of the protein kinase A (PKA) and glutamine amide transfer motifs in the *URA7*-encoded CTP synthetase protein sequence. The site involved in CTP inhibition is also denoted. The numbers on the top of the diagram denote the amino acid positions for each motif in the CTP synthetase protein. The Ser^{424} within the protein kinase A motif that was mutated to an alanine residue is denoted in the figure.

the stimulation of activity and causes a decrease in the sensitivity to CTP product inhibition (*15*, *17*). In addition, phosphorylation of CTP synthetase by protein kinases A and C facilitates the nucleotide-dependent tetramerization of the enzyme (*13*).

Identification of the phosphorylation sites in CTP synthetase is necessary for gaining information about the physiological significance of enzyme phosphorylation. In this work, we examined the hypothesis that amino acid residue Ser^{424} contained in a protein kinase A sequence motif in the *URA7*-encoded CTP synthetase (Figure 1) is the target site for protein kinase A. A synthetic peptide containing the protein kinase A sequence motif was a substrate for protein kinase A and inhibited the phosphorylation of CTP synthetase by protein kinase A. A Ser^{424} → Ala (S424A) mutant enzyme was not phosphorylated by protein kinase A in vivo and in vitro. Purified S424A mutant CTP synthetase exhibited altered kinetic properties and increased sensitivity to CTP product inhibition.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were reagent grade. Growth medium supplies were purchased from Difco Laboratories. Restriction endonucleases, modifying enzymes, and recombinant Vent DNA polymerase with 5′- and 3′-exonuclease activity and the DNA size ladder used for agarose gel electrophoresis were purchased from New England Biolabs. Oligonucleotides were prepared commercially by Genosys Biotechnologies, Inc. The QuikChange site-directed mutagenesis kit was purchased from Stratagene. The Prism DyeDeoxy DNA sequencing kit was obtained from Applied Biosystems. Nucleotides, L-glutamine, 5-fluroorotic acid, phenylmethanesulfonyl fluoride, benzamide, aprotinin, leupeptin, pepstatin, nitrocellulose paper, casein, and bovine serum albumin were purchased from Sigma. The protein kinase A catalytic subunit (bovine heart) was purchased from Promega. Peptides SLGRKDSHSA (PKA1) and LEHSSMKCRR (PKC2) were synthesized and purified commercially by Bio-Synthesis, Inc. The protein assay reagent, electrophoresis reagents, and immunochemical reagents were purchased from Bio-Rad. Radiochemicals and EN^3-HANCE spray were purchased from NEN Life Science Products. Scintillation counting supplies and acrylamide solutions were from National Diagnostics.

Strains, Plasmids, and Growth Conditions. The strains and plasmids used in this work are listed in Table 1. CTP synthetase was expressed in *S. cerevisiae* strain OK8 (*5*). Strain OK8 has mutations in both the *URA7* and *URA8* genes, which are two duplicate genes encoding CTP synthetase (*1*, *5*). Growth of this strain is dependent on a plasmid bearing either the *URA7* or the *URA8* gene (*5*). Methods for growth and analysis of yeast were performed as described previously (*18, 19*). Yeast cultures were grown in complete synthetic medium without inositol (*20*) containing 2% glucose at 30 °C. Cells were also grown on YEPA medium (1% yeast extract, 2% peptone, and 2% acetate). Plasmid maintenance and amplifications were performed in *Escherichia coli* strain DH5α. *E. coli* cells were grown in LB medium [1% tryptone, 0.5% yeast extract, and 1% NaCl (pH 7.4)] at 37 °C. Ampicillin (100 μg/mL) was added to cultures carrying plasmids. Media were supplemented with either 2% (yeast) or 1.5% (*E. coli*) agar for growth on plates. Yeast cell numbers in liquid media were determined by microscopic examination with a hemacytometer or spectrophotometrically at an absorbance of 600 nm.

DNA Manipulations, Amplification of DNA by PCR,[1] *and DNA Sequencing.* Plasmid and genomic DNA preparation, restriction enzyme digestion, and DNA ligations were performed by standard methods (*19*). The transformations of yeast (*21*, *22*) and *E. coli* (*19*) were performed as described previously. Conditions for the amplification of DNA by PCR were optimized as described previously (*23*). DNA sequencing reactions were performed with the Prism DyeDeoxy Terminator Cycle sequencing kit and analyzed with an automated DNA sequencer.

Construction of Plasmids. Mutagenesis of Ser^{424} was performed to examine the hypothesis that this site is

[1] Abbreviation: PCR, polymerase chain reaction.

phosphorylated by protein kinase A. The codon for Ser[424] in the *URA7*-encoded CTP synthetase was changed to an alanine codon by site-directed mutagenesis. The *URA7*^{S424A} mutation was constructed by PCR with the QuikChange site-directed mutagenesis kit with plasmid pDO178 as the template. Plasmid pDO178 contains the *URA7* open reading frame in pBlueScript II (*14*). The primers for the mutation, 5′-GTTGGGAAGAAAAGAtgcgCATTCGGCAGAATTT-3′ and its complement, incorporated an *Fsp*I restriction site. This silent restriction site was used to identify plasmids with the correct mutation. The mutated gene was completely sequenced to verify that no additional mutations were made. The wild-type and S424A mutant alleles of *URA7* were released from pDO178 by digestion with *Not*I and *Pst*I. These 1.8 kb fragments of the wild-type and mutant alleles were then ligated into plasmid pDO105 digested with *Not*I and *Pst*I to form the multicopy shuttle vectors pTP1 and pTP2, respectively. Plasmid pDO105 is a multicopy expression vector containing the *ADH1* promoter (*14*). Single-copy expression plasmids were constructed by subcloning the same wild-type and mutant allele fragments into plasmid pDO120 to form plasmids pTP3 and pTP4, respectively. Plasmid pDO120 contains the *ADH1* promoter and was constructed by subcloning the 1.58 kb *Eco*RI−*Pst*I fragment of pDO105 into the single-copy plasmid YCpLac111 (*24*). The *ura7 ura8* double mutant strain OK8, bearing plasmid pFL44S-*URA7* (*5*), was transformed to leucine prototrophy with the *ADH1* expression plasmids containing the wild-type and S424A mutant alleles of *URA7*. Plasmid pFL44S-*URA7* was subsequently selected against with 5-fluroorotic acid by plasmid shuffle (*25*). Cells were then examined to verify that they regained uracil auxotrophy. The presence of the *ADH1* expression vectors containing the wild-type and mutant alleles of *URA7* was verified by re-isolation in *E. coli* and restriction analysis. We used the *ADH1* promoter for enzyme expression to obviate regulation mediated by the native *URA7* promoter. A multicopy plasmid was chosen for enzyme overexpression to facilitate the purification of the wild-type and mutant enzymes. The single-copy plasmid was used to approximate normal expression of the enzymes and to examine the effects of the S424A mutation in growing cells. The mutant and wild-type alleles were expressed on multicopy and single-copy plasmids in a *ura7 ura8* double mutant to obviate any effects due to the native *URA7*- and *URA8*-encoded enzymes.

In Vivo Labeling of CTP Synthetase. Cells bearing single-copy plasmids containing the wild-type and S424A mutant *URA7* alleles were used to examine the effect of the *RAS*/cAMP pathway on the phosphorylation of CTP synthetase. Exponential phase cells grown in YEPA medium were labeled in low-phosphate (*26*) YEPA medium with $^{32}P_i$ (0.3 mCi/mL) and [^{14}C(U)]-L-amino acids (5 μCi/mL) for 3 h. Glucose was added to a final concentration of 5% to activate the *RAS*/cAMP pathway (*27*). At the indicated time intervals, labeled cells were harvested by centrifugation and washed with phosphate-buffered saline. Cells were disrupted with glass beads in radioimmune precipitation lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS] containing protease inhibitors (0.5 mM phenylmethanesulfonyl fluoride, 1 mM benzamide, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 5 μg/mL pepstatin) and phosphatase inhibitors (10 mM NaF, 5 mM β-glycerophosphate, and 1 mM sodium vanadate) (*28*). CTP synthetase was immunoprecipitated from the cell lysate with anti-*URA7*-encoded CTP synthetase IgG antibodies (*4*) as previously described (*28*). CTP synthetase was dissociated from the enzyme−antibody complex (*28*), and the amount of label incorporated into the enzyme was determined by scintillation counting.

Extraction and Mass Analysis of Nucleotides. Cells expressing the wild-type and S424A mutant *URA7*-encoded CTP synthetases from single-copy plasmids were grown to the exponential phase of growth. Cellular nucleotides were extracted (*1*) and were analyzed by high-performance liquid chromatography (*5*).

Purification of Wild-Type and S424A Mutant CTP Synthetases. Cells expressing the wild-type and S424A mutant *URA7*-encoded CTP synthetases from multicopy plasmids were used for enzyme purification. The CTP synthetases were purified by ammonium sulfate fractionation of the cytosolic fraction followed by chromatography with Sephacryl 300 HR, Q-Sepharose, Affi-Gel Blue, and Superose 6 as described by Yang et al. (*4*).

Electrophoresis and Immunoblotting. SDS−polyacrylamide gel electrophoresis (*29*) was performed with 10% slab gels. Molecular mass standards were phosphorylase b (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa). Proteins on SDS−polyacrylamide gels were stained with Coomassie Blue or with silver. Immunoblot assays were performed with IgG anti-*URA7*-encoded (*4*) CTP synthetase antibodies as described previously (*30*). The density of the *URA7*-encoded CTP synthetase bands on immunoblots was quantified by scanning densitometry. Immunoblot signals were in the linear range of detectability.

Phosphorylation of CTP Synthetase with Protein Kinase A. Phosphorylation reactions were followed for 10 min at 30 °C in a total volume of 40 μL. Pure wild-type and S424A mutant CTP synthetases were incubated with 50 mM Tris-HCl (pH 8.0), 60 mM dithiothreitol, 50 μM [γ-^{32}P]ATP (5 μCi/nmol), 10 mM $MgCl_2$, and the indicated concentrations of the bovine heart protein kinase A catalytic subunit (*15*). The bovine heart protein kinase A catalytic subunit is structurally and functionally similar to the *S. cerevisiae* protein kinase A catalytic subunit (*31*). The protein kinase A preparation used in our studies was judged to be pure as determined by SDS−polyacrylamide gel electrophoresis. At the end of the phosphorylation reactions, samples were treated with 2× Laemmli's sample buffer (*29*), followed by SDS−polyacrylamide gel electrophoresis, immunoblot analysis, and autoradiography. The extent of incorporation of phosphate into CTP synthetase was determined by scintillation counting of phosphorylated enzyme excised from immunoblots. Alternatively, the protein kinase A phosphorylation reactions were performed with unlabeled ATP. Following incubation with protein kinase A, the reaction mixtures were diluted 5-fold and CTP synthetase activity was measured spectrophotometrically as described below.

Tetramerization of CTP Synthetase. The ATP- and UTP-dependent tetramerization of CTP synthetase was analyzed by Superose 6 gel filtration chromatography as described by Pappas et al. (*13*). The CTP synthetase protein in column

fractions was quantified by scanning densitometry of silver-stained SDS−polyacrylamide gels (*13*).

Enzyme Assays, Protein Determination, and Analysis of Kinetic Data. CTP synthetase activity was determined by measuring the rate of 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), 2 mM UTP, 2 mM ATP, 2 mM L-glutamine, 0.1 mM GTP, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and an appropriate dilution of enzyme protein in a total volume of 0.2 mL. Protein kinase A activity was measured by following the phosphorylation of the CTP synthetase synthetic peptide SLGRKDSHSA (PKA1) with [γ-^{32}P]ATP (4 μCi/nmol) for 10 min at 30 °C. The reaction mixture contained 50 mM Tris-HCl buffer (pH 8.0), 50 μM ATP, 10 mM $MgCl_2$, 60 mM dithiothreitol, and the indicated concentrations of peptide substrate. Alternatively, protein kinase A activity was measured with casein (50 μg/mL) as the substrate. Loading samples onto phosphocellulose filter paper terminated reactions. The filters were washed with 75 mM phosphoric acid and subjected to scintillation counting. Enzyme assays were performed in triplicate with an average standard deviation of ±3%. All assays were linear with time and protein concentration. A unit of CTP synthetase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product per minute. A unit of protein kinase A activity was defined in nanomoles of product formed per minute using casein as the substrate. The protein was assessed by the method of Bradford (*32*) using bovine serum albumin as the standard. Kinetic data were analyzed according to the Michaelis−Menten and Hill equations using the EZ-FIT Enzyme Kinetic Model Fitting Program.

RESULTS

A CTP Synthetase Synthetic Peptide Containing a Protein Kinase A Sequence Motif Is a Substrate for Protein Kinase A and Inhibits the Phosphorylation of CTP Synthetase. In vitro, protein kinase A phosphorylates *URA7*-encoded CTP synthetase at a serine residue with a stoichiometry of one phosphorylation site per enzyme subunit (*15*). Examination of the deduced protein sequence of the *URA7*-encoded CTP synthetase revealed that the enzyme has a potential phosphorylation site at Ser^{424} within the protein kinase A sequence motif RKDS (Figure 1). A peptide, SLGRKDSHSA (PKA1), containing this motif was synthesized on the basis of the deduced protein sequence of CTP synthetase. We examined whether this peptide could serve as a substrate for protein kinase A. Protein kinase A catalyzed the phosphorylation of this peptide (Figure 2A). The dependence of protein kinase A activity on the peptide followed saturation kinetics with V_{max} and K_m values of 12.6 nmol min^{-1} mg^{-1} and 30 μM, respectively. The protein kinase A activity using the synthetic peptide as a substrate was about 25% of the activity obtained when casein was used as the substrate using saturating concentrations of substrates. The peptide LEHSSMKCRR (PKC2) was synthesized and used as a negative control. This peptide was also based on the deduced protein sequence of CTP synthetase and contains a putative protein kinase C target site. As expected, this peptide did not serve as a substrate for protein kinase A (Figure 2A).

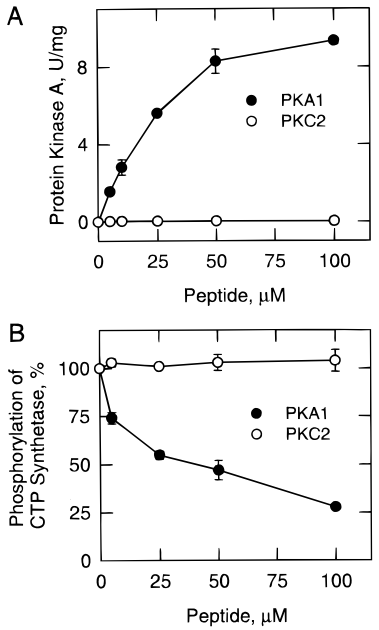

FIGURE 2: CTP synthetase synthetic peptide containing a protein kinase A sequence motif is a substrate for protein kinase A and inhibits the phosphorylation of CTP synthetase. (A) Protein kinase A activity was measured as a function of the concentration of the CTP synthetase synthetic peptides SLGRKDSHSA (PKA1) and LEHSSMKCRR (PKC2). (B) Purified wild-type CTP synthetase (0.4 μg) was incubated with 1 unit/mL protein kinase A for 10 min using [γ-^{32}P]ATP in the absence and presence of the indicated concentrations of the PKA1 and PKC2 peptides. Following the phosphorylation incubations, samples were subjected to SDS−polyacrylamide gel electrophoresis, immunoblot analysis, and autoradiography. The extent of incorporation of phosphate into CTP synthetase was determined by scintillation counting of phosphorylated enzyme excised from the immunoblot. The values reported were the average of three separate experiments ± the standard deviation.

We next examined whether the peptide PKA1 was an inhibitor of protein kinase A activity when purified wild-type CTP synthetase was used as a substrate. CTP synthetase was phosphorylated with protein kinase A in the absence and presence of the synthetic peptide. The peptide inhibited the phosphorylation of the enzyme in a dose-dependent manner (Figure 2B). The IC_{50} value for the peptide was 45 μM. The control peptide PKC2 did not affect the phosphorylation of CTP synthetase by protein kinase A (Figure 2B).

Characterization of the CTP Synthetase S424A Mutant. Cells bearing multicopy and single-copy plasmids containing the wild-type and S424A mutant alleles of the *URA7* gene exhibited similar growth rates when grown vegetatively at 30 °C in rich YEPD and in minimal synthetic media. No morphological differences were observed in the cells bearing the S424A mutation in the *URA7*-encoded CTP synthetase. Immunoblot analysis showed that antibodies directed against the wild-type CTP synthetase protein recognized the S424A mutant CTP synthetase (Figure 3A). Moreover, scanning densitometry of the immunoblot shown in Figure 3A indicated that there were no differences in the amounts of the wild-type and S424A mutant CTP synthetase protein levels expressed in cells from the single-copy plasmids (Figure 3A,B). Thus, the S424A mutation in the *URA7* gene did not affect the functional expression of the enzyme. However, the specific activity of the S424A mutant CTP synthetase in the cell extract was 40% lower than that of the wild-type enzyme (Figure 3B).

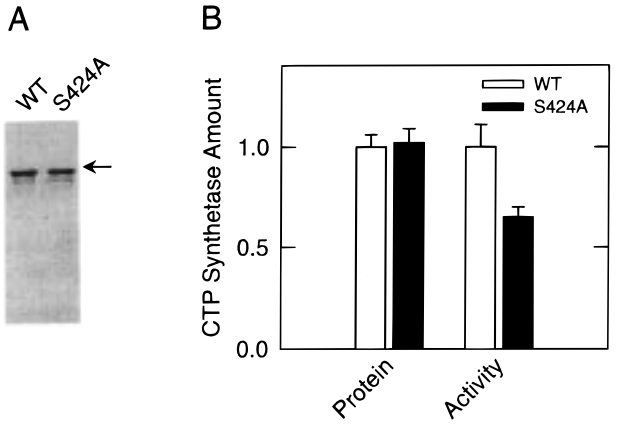

FIGURE 3: CTP synthetase protein and activity levels in cells bearing the S424A mutation in CTP synthetase. Cells expressing the indicated S424A mutant and wild-type (WT) CTP synthetase enzymes from a single-copy plasmid were grown in complete synthetic medium to the exponential phase of growth. Cell extracts were prepared and assayed for CTP synthetase protein by immunoblot analysis and for activity as described in Experimental Procedures. (A) Immunoblot of wild-type and S424A mutant CTP synthetase. The arrow in the figure denotes the position of CTP synthetase. (B) The amounts of CTP synthetase protein and activity found in cells bearing the wild-type enzyme were set a 1. The values that are reported were the average of three separate experiments ± the standard deviation. The specific activities of CTP synthetase in cells bearing the wild-type and S424A mutant enzymes were 16.7 ± 2 and 11 ± 0.9 milliunits/mg, respectively.

Protein kinase A is the principle mediator of signals transmitted through the *RAS*/cAMP pathway in *S. cerevisiae* (*33*, *34*). We examined the effect of the S424A mutation on the protein kinase A-mediated phosphorylation of CTP synthetase in cells that were activated in the *RAS*/cAMP pathway. The *RAS*/cAMP pathway is activated by the addition of glucose to nonfermenting cells (*27*, *34*). Glucose triggers a transient increase in cAMP levels, protein kinase A activity, and the extent of phosphorylation of enzymes known to be substrates for protein kinase A (*27*, *34*). Cells expressing the wild-type and S424A mutant CTP synthetase alleles on the single-copy plasmid were grown in YEPA medium to attenuate the *RAS*/cAMP pathway (*27*). Cells were then incubated for 3 h with $^{32}P_i$ for detection of phosphorylated CTP synthetase and [^{14}C(U)]-L-amino acids to normalize for the amount of CTP synthetase that was isolated. The ratio of counts per minute of ^{32}P incorporated into CTP synthetase to the counts of ^{14}C incorporated into CTP synthetase was used to examine the extent of phosphorylation (*15*). Glucose (5%) was then added to the growth medium to activate protein kinase A (*27*). The wild-type and S424A mutant CTP synthetases were isolated by immunoprecipitation, and the amount of each label incorporated into the enzymes was determined. The activation of the *RAS*/cAMP pathway resulted in a time-dependent increase (3-fold) in the extent of phosphorylation of the wild-type CTP synthetase (Figure 4). On the other hand, the phosphorylation of the S424A mutant CTP synthetase was not affected by the activation of the *RAS*/cAMP pathway (Figure 4).

The effect of the S424A mutation on the cellular concentration of nucleoside triphosphates was examined. Cells bearing the wild-type and S424A mutant CTP synthetase expressed from the single-copy plasmid were grown in complete synthetic medium to the exponential phase of growth, and nucleotides were extracted and then analyzed by high-performance liquid chromatography. The cellular concentration of CTP, as well as those of other nucleotides, including UTP, ATP, and GTP, were not significantly altered in cells bearing the S424A mutant CTP synthetase when compared with those of the control cells (Table 2).

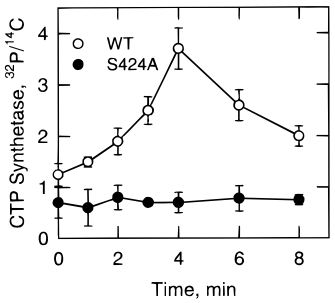

FIGURE 4: Effect of the S424A mutation in CTP synthetase on the *RAS*/cAMP-mediated phosphorylation of CTP synthetase. Cultures (10 mL) of cells expressing the indicated S424A mutant and wild-type (WT) CTP synthetase enzymes from a single-copy plasmid were grown in YEPA medium to the exponential phase of growth (1×10^7 cells/mL). Cells were harvested by centrifugation, washed with low-phosphate YEPA medium, and resuspended in 1 mL of the same medium. Cells were then labeled with $^{32}P_i$ and [^{14}C(U)]-L-amino acids for 3 h. Glucose was then added to a final concentration of 5% to activate the *RAS*/cAMP pathway. CTP synthetase was immunoprecipitated from cells 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 counts per minute of ^{32}P incorporated into CTP synthetase relative to the counts per minute of ^{14}C incorporated into CTP synthetase. The values reported were the average of three separate experiments ± the standard deviation.

Table 2: Cellular Concentrations of Nucleotides in Cells Carrying the S424A Mutant and Wild-Type CTP Synthetases

	cellular concentration (mM)	
nucleotide[a]	S424A	WT
ATP	1.01 ± 0.07	1.00 ± 0.04
UTP	0.33 ± 0.02	0.32 ± 0.02
GTP	0.27 ± 0.02	0.27 ± 0.02
CTP	0.33 ± 0.02	0.30 ± 0.01

[a] Cells expressing the S424A mutant and the wild-type (WT) CTP synthetase from a single-copy plasmid were grown to the exponential phase of growth. Nucleotides were extracted and analyzed by high-performance liquid chromatography as described in Experimental Procedures. The values reported were the average of three separate experiments ± the standard deviation.

Characterization of the Purified S424A Mutant CTP Synthetase. The wild-type and S424A mutant CTP synthetases were purified from cells overexpressing these enzymes. Analysis by SDS−polyacrylamide gel electrophoresis indicated that these enzymes were purified to apparent homogeneity (Figure 5). The specific activity of the purified S424A mutant enzyme (1.8 units/mg) was 20% lower than that of the purified wild-type enzyme (2.3 units/mg). Although this difference was relatively small, it could be reproduced in three separate purified enzyme preparations. The lower specific activity of the mutant enzyme was not due to proteolysis as evidenced by the analysis of SDS−polyacrylamide gels containing the pure enzymes. CTP synthetase activity is dependent on its ATP- and UTP-dependent oligomerization from a dimer to a tetramer (*13*). The S424A mutation did not affect this property (data not shown).

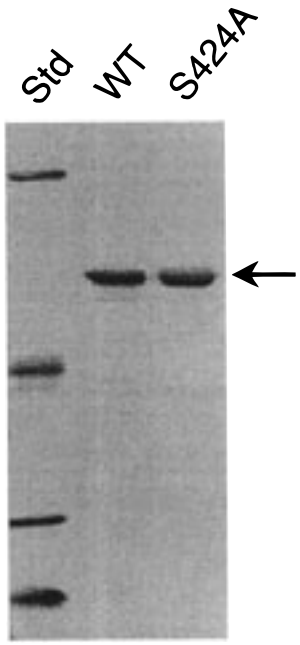

FIGURE 5: SDS−polyacrylamide gel electrophoresis of purified S424A mutant and wild-type CTP synthetases. Purified S424A mutant and wild-type CTP synthetases were subjected to SDS−polyacrylamide gel electrophoresis and stained with Coomassie blue as described in Experimental Procedures. The protein molecular mass standards (Std) from top to bottom are phosphorylase b (92.5 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa). The position of CTP synthetase is denoted in the figure.

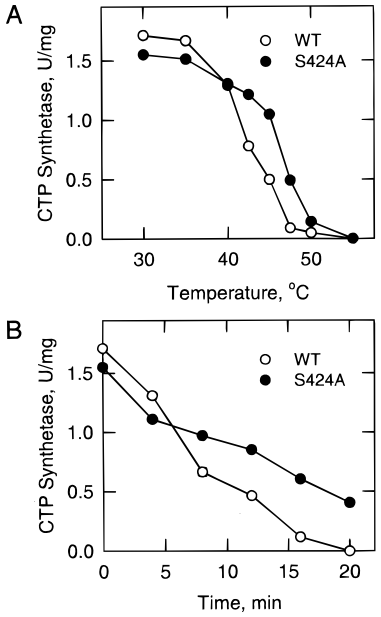

FIGURE 6: Effect of the S424A mutation in CTP synthetase on the temperature stability of CTP synthetase. (A) Samples of purified S424A mutant and wild-type (WT) CTP synthetases were incubated at the indicated temperatures for 10 min. (B) Samples of purified S424A mutant and wild-type (WT) CTP synthetases were incubated at 45 °C for the indicated time intervals. After the incubations, the samples were cooled on ice for 10 min followed by the measurement of CTP synthetase activity at 30 °C. The values that are reported were the average of three separate experiments ± the standard deviation.

We considered the possibility that the S424A mutant CTP synthetase protein was more sensitive to thermal denaturation. The purified wild-type and mutant enzymes were incubated for 10 min at temperatures ranging from 30 to 60 °C. After incubation, the enzymes were cooled in an ice bath for 10 min to allow for protein renaturation followed by the measurement of CTP synthetase activity at 30 °C. Both CTP synthetases were labile at temperatures above 30 °C with total inactivation at 60 °C (Figure 6A). However, the S424A mutant CTP synthetase was more resistant to thermal inactivation at temperatures below 60 °C when compared with the wild-type enzyme. The $t_{1/2}$ values for the inactivation of the S424A mutant and wild-type CTP synthetases at 45 °C were 13 and 7 min, respectively (Figure 6B). Thus, the lower specific activity of the mutant enzyme was not due to thermal denaturation. In fact, the mutation afforded greater stability to thermal denaturation.

The phosphorylation of the pure S424A mutant CTP synthetase by protein kinase A was examined. Samples of the wild-type and S424A mutant CTP synthetases were incubated with protein kinase A and ^{32}P-labeled ATP. After the phosphorylation reactions, samples were subjected to SDS−polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. As described previously (*15*), autoradiography of the nitrocellulose paper showed that the wild-type CTP synthetase was a substrate for protein kinase A. Protein kinase A activity was dependent on the concentration of CTP synthetase (Figure 7A) and on the time of the reaction (Figure 7B). On the other hand, protein kinase A did not catalyze the incorporation of the γ-phosphate of ^{32}P-labeled ATP into the purified S424A mutant CTP synthetase (Figure 7). The amounts of the wild-type and S424A mutant CTP synthetases on the nitrocellulose papers were confirmed by immunoblot analysis. As described previously (*15*), the phosphorylation of the wild-type CTP synthetase with protein kinase A was accompanied by a dose-dependent stimulation of CTP synthetase activity (Figure 8). However, CTP synthetase activity of the S424A mutant was not affected by incubation with increasing concentrations of protein kinase A (Figure 8).

The in vitro phosphorylation of the purified wild-type CTP synthetase by protein kinase A stimulates CTP synthetase activity by a mechanism that includes an increase in the V_{max} of the reaction with respect to ATP and UTP, and a decrease in the K_m value for ATP (*15*). Kinetic experiments were performed with the purified S424A mutant CTP synthetase to examine the hypothesis that the mutation would have the opposite effects on the kinetics of the enzyme. These experiments were performed with saturating concentrations of glutamine, GTP, and magnesium ions. The effect of the S424A mutation on the dependence of CTP synthetase activity on ATP was examined using a subsaturating concentration of UTP. The S424A mutation resulted in a decrease in the apparent V_{max} of the reaction and an increase in the apparent K_m for ATP when compared with those of the wild-type enzyme, and when compared with those of the wild-type enzyme phosphorylated with protein kinase A (Figure 9A and Table 3). The specificity constant for ATP for the S424A mutant CTP synthetase was 44 and 76% lower than that of the wild-type enzyme and the phosphorylated wild-type enzyme, respectively. The effect of the S424A mutation in CTP synthetase on the kinetics of the enzyme toward ATP was less evident when activity was measured with a saturating concentration of UTP.

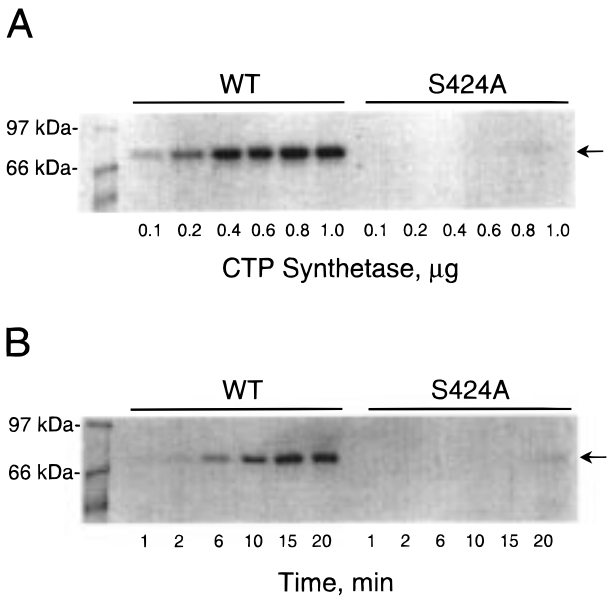

FIGURE 7: Effect of the S424A mutation on the phosphorylation of CTP synthetase by protein kinase A. (A) Protein kinase A (0.25 unit/mL) and [γ-^{32}P]ATP were incubated with the indicated concentrations of purified S424A mutant and wild-type (WT) CTP synthetases for 10 min. (B) Purified S424A mutant (0.8 μg) and wild-type CTP synthetases (0.8 μg) were incubated with protein kinase A (0.25 unit/mL) and [γ-^{32}P]ATP for the indicated time intervals. Following the phosphorylation incubations, samples were subjected to SDS−polyacrylamide gel electrophoresis, immunoblot analysis, and autoradiography. Portions of the autoradiograms showing the positions of CTP synthetase and protein molecular mass standards are shown. The arrows in the figure denote the position of CTP synthetase.

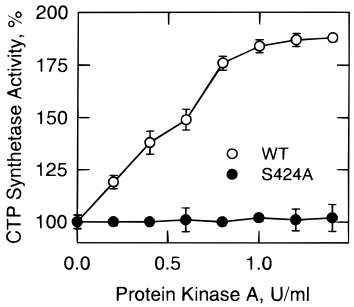

FIGURE 8: Effect of the S424A mutation on the stimulation of CTP synthetase activity by protein kinase A. Purified S424A mutant and wild-type (WT) CTP synthetases were incubated with the indicated amounts (U is unit) of protein kinase A for 10 min. Following the incubations, samples were diluted 5-fold, and CTP synthetase activity was measured as described in the text using 0.5 mM ATP and 0.2 mM UTP as substrates. The values reported were the average of three separate experiments ± the standard deviation.

The effect of the S424A mutation on the dependence of CTP synthetase activity on UTP was examined using a saturating concentration of ATP. The mutation in CTP synthetase resulted in a decrease in the apparent V_{max} value and an increase in the apparent K_m value for UTP when compared with those of the wild-type enzyme, and when compared with those of the wild-type enzyme phosphorylated with protein kinase A (Figure 9B and Table 3). The specificity constant for UTP for the S424A mutant CTP synthetase was 41 and 66% lower when compared with those of the wild-type enzyme and the phosphorylated wild-type enzyme, respectively. The mutation did not affect the Hill number (1.45) for UTP. The effects of the S424A mutation

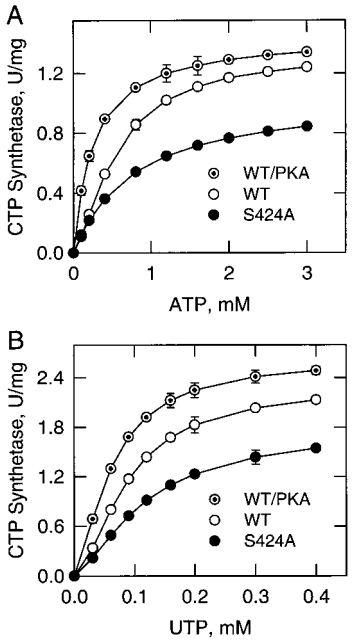

FIGURE 9: Effect of the S424A mutation on the kinetics of CTP synthetase activity with respect to ATP and UTP. Purified S424A mutant and wild-type CTP synthetases were incubated with 1 unit/mL protein kinase A for 10 min. Following the incubations, samples were diluted 5-fold in assay buffer. A sample of the purified wild-type enzyme was not treated with protein kinase A. S424A mutant, untreated wild-type (WT), and phosphorylated wild-type (WT/PKA) CTP synthetase activities were measured as a function of the concentration of ATP using 0.1 mM UTP (A) and as a function of the concentration of UTP using 2 mM ATP (B). The concentrations of glutamine, GTP, and $MgCl_2$ were maintained at 2, 0.1, and 10 mM, respectively. The values reported were the average of three separate experiments ± the standard deviation.

on the kinetic properties of the enzyme were less evident when activity was measured with a subsaturating ATP concentration. These data indicated that the phosphorylation of CTP synthetase by protein kinase A at Ser^{424} was responsible for the alterations in the kinetic properties of the enzyme.

Phosphorylation of the wild-type purified enzyme with protein kinase A results in a decrease in the sensitivity of the enzyme to product inhibition by CTP (*15*). We examined the hypothesis that the S424A mutation in CTP synthetase would render the enzyme more sensitive to CTP product inhibition. The S424A mutant CTP synthetase activity was measured in the absence and presence of CTP. These experiments were performed using subsaturating concentrations of ATP and UTP and saturating concentrations of glutamine, GTP, and magnesium ions. The S424A mutant enzyme was indeed more sensitive to inhibition by CTP when compared with the wild-type enzyme, and when compared with the wild-type enzyme phosphorylated with protein kinase A (Figure 10A). The inhibition constant for CTP of the S424A mutant CTP synthetase was 48 and 63% lower than the inhibition constant for CTP of the wild-type enzyme and the phosphorylated wild-type enzyme, respectively (Table 3).

The effect of 0.3 mM CTP on the kinetics of CTP synthetase activity toward UTP was examined for the S424A mutant enzyme using a subsaturating concentration of ATP and saturating concentrations of the other substrates in the reaction (Figure 10B). The apparent V_{max} value of the S424A

Table 3: Kinetic Constants for the Wild-Type and S424A Mutant CTP Synthetases

	S424A				WT				WT/PKA			
substrate or inhibitor	V_{max}(app) (units/mg)	K_m(app) (mM)	V_{max}/K_m	IC_{50} (mM)	V_{max}(app) (units/mg)	K_m(app) (mM)	V_{max}/K_m	IC_{50} (mM)	V_{max}(app) (units/mg)	K_m(app) (mM)	V_{max}/K_m	IC_{50} (mM)
ATP[a]	1.06	0.77	1.4		1.35	0.54	2.5		1.45	0.25	5.8	
UTP[b]	1.81	0.12	15.1		2.30	0.09	25.5		2.65	0.06	44.2	
UTP[c]	0.61	0.23	2.7		1.32	0.23	5.7		1.93	0.26	7.5	
CTP[d]				0.13				0.25				0.35

[a] Kinetic constants determined with 0.1 mM UTP. [b] Kinetic constants determined with 2 mM ATP. [c] Kinetic constants determined with 1 mM ATP in the presence of 0.3 mM CTP. [d] Kinetic constant determined with 0.1 mM UTP and 1 mM ATP.

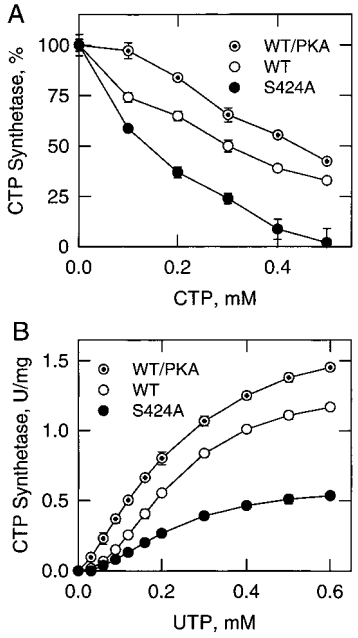

FIGURE 10: Effect of the S424A mutation on the inhibition of CTP synthetase activity by CTP and on the effect of CTP on the kinetics of CTP synthetase activity with respect to UTP. Purified S424A mutant and wild-type CTP synthetases were incubated with 1 unit/mL protein kinase A for 10 min. Following the incubations, samples were diluted 5-fold in assay buffer. A sample of the purified wild-type enzyme was not treated with protein kinase A. (A) S424A mutant, untreated wild-type (WT), and phosphorylated wild-type (WT/PKA) CTP synthetase activities were measured with 0.1 mM UTP in the absence and presence of the indicated concentrations of CTP using 1 mM ATP. The concentrations of glutamine, GTP, and $MgCl_2$ were maintained at 2, 0.1, and 10 mM, respectively. (B) S424A mutant, untreated wild-type (WT), and phosphorylated wild-type (WT/PKA) CTP synthetase activities were measured as a function of the concentration of UTP in the presence of 0.3 mM CTP. The concentrations of ATP, glutamine, GTP, and $MgCl_2$ were maintained at 1, 2, 0.1, and 10 mM, respectively. The values reported were the average of three separate experiments ± the standard deviation.

mutant enzyme was 54 and 68% lower than the apparent V_{max} value of the wild-type enzyme and the phosphorylated wild-type enzyme, respectively (Table 3). Thus, the phosphorylation of CTP synthetase by protein kinase A at Ser^{424} was responsible for the decrease in the sensitivity of the enzyme to inhibition by CTP. The apparent K_m value for UTP (Table 3) and the Hill number for UTP (2.1) for the S424A mutant enzyme activity measured in the presence of CTP were similar to those of the wild-type enzyme and the phosphorylated wild-type enzyme. The mechanism of CTP inhibition of the wild-type CTP synthetase involves an increase in the positive cooperativity of the enzyme toward UTP and a decrease in the enzyme affinity for UTP (*4*). Thus, the S424A mutation did not affect the mechanism by which CTP inhibits CTP synthetase activity.

DISCUSSION

The enzyme CTP synthetase plays a major role in the growth and metabolism of eukaryotic organisms. For example, proper regulation of CTP synthetase activity by CTP controls the balance of nucleotide pools (*1*, *4*, *5*, *14*, *35*−*37*) and influences the pathways by which membrane phospholipids are synthesized (*10*, *14*, *38*). Unregulated CTP synthetase activity is a common property of leukemic cells (*39*−*41*) and rapidly growing tumors of liver (*42*), colon (*43*), and lung (*44*). By and large, these observations underscore the importance of studies in understanding the regulation of CTP synthetase activity.

Phosphorylation is a major mechanism by which enzymes are regulated (*45*, *46*), and indeed, the yeast CTP synthetase is regulated by phosphorylation. The *URA7*-encoded CTP synthetase is phosphorylated at multiple sites in vivo (*16*). In vitro studies have shown that CTP synthetase is a substrate for protein kinase A (*15*) and for protein kinase C (*16*, *17*). These phosphorylations appear to stimulate CTP synthetase activity by a common mechanism (*15*, *17*). Yet, phosphopeptide mapping experiments indicate that the protein kinase A and protein kinase C sites in the enzyme differ (*15*, *16*). Results of phosphorylation experiments with dephosphorylated CTP synthetase suggest that the enzyme may be phosphorylated by additional protein kinases and/or a hierarchical phosphorylation sequence may exist (*14*, *15*, *47*). Preliminary studies have shown that the *URA7*-encoded CTP synthetase is also phosphorylated by casein kinase II.[2] However, neither the protein kinase C nor the casein kinase II phosphorylation sites in the enzyme have been identified.

In this work, we addressed the complex phosphorylation of the *URA7*-encoded CTP synthetase through studies to identify the protein kinase A target site in the enzyme. The peptide PKA1, which contained the protein kinase A sequence motif at Ser^{424}, was a substrate for protein kinase A. This peptide also inhibited the phosphorylation of CTP synthetase by protein kinase A. These data supported the conclusion that a protein kinase A target sequence existed within the peptide PKA1 and provided confidence that the protein kinase A phosphorylation site in CTP synthetase may be Ser^{424}. A $Ser^{424} \rightarrow$ Ala mutation in the enzyme was constructed and was used to support this hypothesis. In vivo labeling experiments showed that the phosphorylation state of the S424A mutant CTP synthetase was not affected by the activation of the *RAS*/cAMP pathway. As described

[2] A. Pappas and G. M. Carman, unpublished work.

previously (*15*), the phosphorylation state of the wild-type enzyme increased when the *RAS*/cAMP pathway was activated. These results were consistent with the conclusion that the S424A mutation prevented CTP synthetase from being phosphorylated by protein kinase A in vivo.

We purified the S424A mutant CTP synthetase to apparent homogeneity to further confirm that Ser^{424} was the protein kinase A phosphorylation site in the enzyme. The S424A mutant CTP synthetase behaved like the wild-type enzyme during each step of the purification. Moreover, the S424A mutation did not affect the stability of the enzyme to temperature or the nucleotide-dependent tetramerization of the enzyme. This suggested that the S424A mutation did not have a significant effect on the structure of the protein. The purified S424A mutant enzyme was not phosphorylated and stimulated by protein kinase A.

The S424A mutation in CTP synthetase caused a decrease in the specific activity of the enzyme. The kinetic analysis of the mutant enzyme showed that the S424A mutation caused a decrease in CTP synthetase activity by decreasing the V_{max} of the reaction and elevating the K_m values for ATP and UTP. The mutation also rendered the enzyme more sensitive to CTP product inhibition. The apparent kinetic constants for ATP, UTP, and CTP of the phosphorylated wild-type and S424A mutant enzymes were within the physiological range of these nucleotides. Thus, changes in the phosphorylation state of the enzyme, brought about by the phosphorylation of Ser^{424} by protein kinase A, might be expected to affect CTP synthetase activity in vivo. Yet, the S424A mutation in CTP synthetase did not affect the steady-state levels of the nucleotides involved in the CTP synthetase reaction, including the product CTP. This is perhaps not surprising since the activation of the *RAS*/cAMP pathway in cells caused a transient increase in the phosphorylation state of the wild-type enzyme. Thus, the increased CTP synthetase activity of the wild-type enzyme mediated by protein kinase A phosphorylation may be short-lived in vivo and would not be expected to alter steady-state nucleotide levels. As discussed above, the regulation of CTP synthetase by phosphorylation is complex. The S424A mutant cells may have compensated for the defect in the protein kinase A phosphorylation of CTP synthetase by altering the phosphorylation of the enzyme by other protein kinases. Additional studies will be required before the physiological significance of the phosphorylation of CTP synthetase by protein kinase A can be established. Accordingly, future studies will be directed toward defining the phosphorylation sites in CTP synthetase by the other protein kinases that regulate CTP synthetase. The examination of the effects of these phosphorylations in combination with the protein kinase A phosphorylation of CTP synthetase should help in the elucidation of the complex regulation of CTP synthetase activity by phosphorylation in vivo.

In summary, we have utilized a combination of biochemical and molecular approaches to examine the protein kinase A phosphorylation site in the *URA7*-encoded CTP synthetase. The compilation of the data reported here supported the identification of the protein kinase A phosphorylation site in CTP synthetase as Ser^{424}. This information and the availability of the S424A mutant will permit further defined studies on the regulation of this important enzyme by phosphorylation via protein kinase A. This regulation is likely to represent a mechanism by which the *RAS*/cAMP pathway mediates cell growth and metabolism in yeast.

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