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Review

CTP synthetase and its role in phospholipid synthesis in the yeast *Saccharomyces cerevisiae*

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ABSTRACT

CTP synthetase is a cytosolic-associated glutamine amidotransferase enzyme that catalyzes the ATP-dependent transfer of the amide nitrogen from glutamine to the C-4 position of UTP to form CTP. In the yeast *Saccharomyces cerevisiae*, the reaction product CTP is an essential precursor of all membrane phospholipids that are synthesized via the Kennedy (CDP-choline and CDP-ethanolamine branches) and CDP-diacylglycerol pathways. The *URA7* and *URA8* genes encode CTP synthetase in *S. cerevisiae*, and the *URA7* gene is responsible for the majority of CTP synthesized *in vivo*. The CTP synthetase enzymes are allosterically regulated by CTP product inhibition. Mutations that alleviate this regulation result in an elevated cellular level of CTP and an increase in phospholipid synthesis via the Kennedy pathway. The *URA7*-encoded enzyme is phosphorylated by protein kinases A and C, and these phosphorylations stimulate CTP synthetase activity and increase cellular CTP levels and the utilization of the Kennedy pathway. The *CTPS1* and *CTPS2* genes that encode human CTP synthetase enzymes are functionally expressed in *S. cerevisiae*, and rescue the lethal phenotype of the *ura7Δ ura8Δ* double mutant that lacks CTP synthetase activity. The expression in yeast has revealed that the human *CTPS1*-encoded enzyme is also phosphorylated and regulated by protein kinases A and C.

Contents

1. Introduction

CTP is an essential nucleotide that is synthesized from UTP via the reaction catalyzed by the cytosolic-associated enzyme CTP synthetase [1,2] (Fig. 1). The enzyme catalyzes a complex set of reactions that include the ATP-dependent transfer of the amide nitrogen from glutamine (i.e., glutaminase reaction) to the C-4 position of UTP to generate CTP (Fig. 1). GTP stimulates the glutaminase reaction by accelerating the formation of a covalent glutaminyl enzyme intermediate [2,3]. In the yeast *Saccharomyces cerevisiae*, CTP synthetase is an essential enzyme [4,5] that provides the CTP precursor of the CDP-based intermediates used for the synthesis of membrane phospholipids (Fig. 2). This review summarizes our current understanding of the regulation of CTP synthetase activity, and how this regulation influences the synthesis of membrane phospholipids in *S. cerevisiae*. We will also discuss how studies with yeast have facilitated a greater understanding of the regulation of human CTP synthetase.

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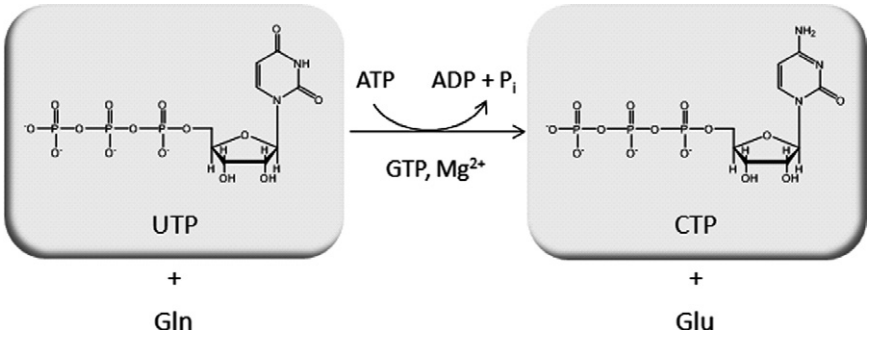

Fig. 1. Reaction catalyzed by CTP synthetase. The figure shows the structures of UTP and CTP, and the reaction catalyzed by CTP synthetase.

2. The central role of CTP in the synthesis of membrane phospholipids

The CTP synthetase reaction product CTP plays an essential role in the synthesis of all membrane phospholipids in *S. cerevisiae* [6–8]. CTP is the direct precursor of the activated, energy-rich phospholipid pathway intermediates CDP-diacylglycerol [9], CDP-choline [10], and CDP-ethanolamine [10] (Fig. 2). CDP-diacylglycerol is the source of the phosphatidyl moiety of the major phospholipids phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine that is synthesized by way of the CDP-diacylglycerol pathway (shown in red color) as well as phosphatidylglycerol, cardiolipin, and phosphatidylinositol [6–8,11] (Fig. 2). The phosphatidylinositol derived from CDP-diacylglycerol also serves as the precursor for yeast sphingolipids [12,13], the D-3, D-4, and D-5 phosphoinositides [7,14–17], and glycosyl phosphatidylinositol anchors [18,19] (Fig. 2). CDP-choline and CDP-ethanolamine are the sources of the hydrophilic head groups of phosphatidylcholine and phosphatidylethanolamine that are synthesized by the CDP-choline and CDP-ethanolamine branches, respectively, of the Kennedy pathway [6–8,11] (Fig. 2, shown in green color).

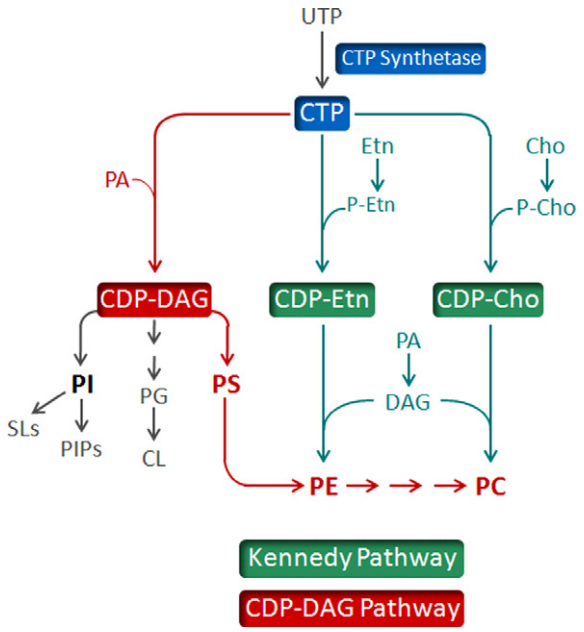

Fig. 2. The role of CTP synthetase in the synthesis of the major phospholipids in *S. cerevisiae*. The pathways shown for the synthesis of phospholipids include the relevant steps discussed in this review. A more comprehensive description of the phospholipid biosynthetic pathways in *S. cerevisiae* may be found in references [7,54]. The abbreviations used are: PA, phosphatidate; Etn, ethanolamine; P-Etn, phosphoethanolamine; CDP-DAG, CDP-diacylglycerol; CDP-Etn, CDP-ethanolamine; CDP-Cho, CDP-choline; PI, phosphatidylinositol; PG, phosphatidylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine, PC, phosphatidylcholine; SLs, sphingolipids; PIPs, polyphosphoinositides. The Kennedy and CDP-DAG pathways are differentiated by color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. CTP synthetases of *S. cerevisiae*

Ozier-Kalogeropoulos and coworkers [4,5] have identified two genes (i.e., *URA7* and *URA8*) that encode CTP synthetase enzymes in *S. cerevisiae*. The coding sequences of the *URA7* and *URA8* genes show 70% identity at the nucleotide level, and their deduced amino acid sequences (i.e., Ura7p and Ura8p) show 78% identity [4,5]. The subunit molecular masses of Ura7p and Ura8p are 64.7 and 64.5 kDa, respectively [4,5]. Neither gene is essential provided that cells possess one functional gene encoding the CTP synthetase enzyme [5]. The *URA7* mRNA is 2-fold more abundant than the *URA8* transcript [5,20,21]. In a *ura8* mutant, CTP levels are 22% lower than in wild-type, whereas the CTP concentration in a *ura7* mutant is 64% lower than in wild-type [5]. Thus, the *URA7*-encoded CTP synthetase is responsible for the majority of the CTP made *in vivo* [5].

The *URA7*- and *URA8*-encoded CTP synthetase enzymes have been purified to homogeneity and characterized with respect to their kinetic and enzymological properties [21,22]. The purification of the enzymes has been facilitated by their overexpression in yeast on multicopy plasmids [21,22]. The purified *URA7*- and *URA8*-encoded CTP synthetases exist as dimers and they oligomerize to tetramers in the presence of the substrates UTP and ATP [21–23]. The two enzymes differ, however, with respect to their kinetic properties. The *URA7*-encoded enzyme shows positive cooperative kinetics when the UTP concentration is varied at a saturating concentration of ATP or when the ATP concentration is varied at a saturating concentration of UTP [22]. In contrast, the *URA8*-encoded CTP synthetase exhibits positive cooperative kinetics when the UTP concentration is varied at subsaturating concentrations of ATP or when the ATP concentration is varied at subsaturating concentrations of UTP [21]. The cellular concentrations of UTP (0.75 mM) and ATP (2.3 mM) are considered to be saturating for both the *URA7*- and *URA8*-encoded enzymes [4,22]. Under these conditions, the *URA8*-encoded CTP synthetase would be expected to exhibit saturation kinetics toward UTP and ATP, and the *URA7*-encoded CTP synthetase would be expected to show positive cooperativity toward the substrates. Thus, it is possible that the activities of both enzymes may be regulated differentially *in vivo* through the cellular concentrations of UTP and ATP [21].

CTP synthetase enzymes have been isolated from both prokaryotic and eukaryotic organisms [2,21,22,24–27]. Moreover, structures for the bacterial [28,29] and human [30] enzymes have been solved. The yeast CTP synthetases have high homology to

bacterial and mammalian CTP synthetase enzymes [28–32]. All CTP synthetases identified from bacteria, parasites, yeast, and mammals contain conserved CTP synthetase and glutamine amide transfer domains that are involved in catalysis (Fig. 3) [28–30,33–39]. The analysis of the *Escherichia coli* structure has shown that CTP synthetase has a novel product inhibition mechanism in which shared substrate and product moieties bind to a single subsite while specificity is conferred by separate sites [34]. The enzymological and kinetic properties of the enzyme from various organisms are similar, although some differences have been noted [25,27].

4. CTP regulates CTP synthetase activity and the synthesis of membrane phospholipids

The *URA7*- and *URA8*-encoded CTP synthetase enzymes are allosterically inhibited by the product CTP [21,22]. This regulation ultimately determines the intracellular concentration of CTP [20,22]. CTP inhibits the CTP synthetase activity by increasing the positive cooperativity of the enzyme for UTP, and at the same time, decreasing the affinity (as reflected in an increase in K_m) for UTP [21,22]. This inhibition mechanism is also true for the CTP synthetases from *E. coli* [2] and rat liver [26]. Because CTP and UTP have distinct binding sites on CTP synthetase [32,40], it is likely that CTP does not compete with UTP for the active site.

CTP synthetase mutants defective in CTP product inhibition have been isolated from Chinese hamster ovary cells [41]. The mutants were selected for their resistance to the growth inhibitory effects of the chemotherapeutic drugs arabinosyl cytosine and 5-fluorouracil [41]. Sequence analysis of the CTP synthetase gene from these mutants has revealed that most of the mutations are clustered within a stretch of 14 amino acids [41]. The most frequent mutations in the clustered region of the enzyme are glutamate-to-lysine and histidine-to-lysine changes [41]. The clustered sites are in a highly conserved region of the CTP synthetases from human cells [42], *E. coli* [31], *Chlamydia trachomatis* [43], *Bacillus subtilis* [44], and *S. cerevisiae* [4,5]. These amino acids correspond to Glu^{161} and His^{233} in the *URA7*- and *URA8*-encoded CTP synthetases. The codons for Glu^{161} and His^{233} in the *URA7* and *URA8* genes have been changed to lysine codons individually or in combination by site-directed mutagenesis, and the effect of these mutations on the balance of pyrimidine nucleotide pools in cells have been examined [45]. Between the two mutations, E161K showed a more significant effect on CTP synthetase activity when compared with the H233K mutation [45]. The *URA7*- and *URA8*-encoded CTP synthetases containing the E161K mutation are less sensitive to CTP product inhibition and are resistant to growth inhibition of cyclopentenylcytosine [45], another drug used for cancer chemotherapy [46–49]. Consequently, cells expressing the E161K mutant enzymes have greater intracellular level (6–15-fold) of CTP when compared with cells that express the wild-type forms of the enzyme [45].

In addition to elevated CTP levels, cells expressing the E161K mutation show alterations in the synthesis of membrane phospholipids [45]. These cells exhibit an increase in the synthesis of phosphatidylcholine, phosphatidylethanolamine, and phosphatidate, and a decrease in the synthesis of phosphatidylserine [45]. The increase in phosphatidylcholine synthesis has been ascribed to an increase in the utilization of the CDP-choline branch of the Kennedy pathway [45] (Fig. 2). The increased utilization of the Kennedy pathway in response to elevated CTP levels has been attributed to the stimulation of phosphocholine cytidylyltransferase activity, the enzyme that catalyzes the rate-limiting penultimate step in the Kennedy pathway (Fig. 1) [20]. The stimulation of this activity is simply due to increased substrate availability of CTP [20,50]. In wild-type cells, the apparent K_m of CTP (1.4 mM) for the phosphocholine cytidylyltransferase [51] is 2-fold higher than the cellular concentration of CTP (0.7 mM) [20]. Thus, the activity of the cytidylyltransferase enzyme would be sensitive to the elevation of cellular CTP levels [20]. Indeed, the overexpression of CTP synthetase activity results in an increase in the cellular concentration of CDP-choline [20,50]. Likewise, the K_m of CTP (1 mM) for the CDP-diacylglycerol synthase [52] is higher than the cellular concentration of CTP in wild-type cells, and the overexpression of CTP synthetase activity results in an increase in the cellular concentration of CDP-diacylglycerol [20]. However, this does not result in a greater utilization of the CDP-diacylglycerol pathway for phosphatidylcholine synthesis [20]. In contrast to the phosphocholine cytidylyltransferase reaction, the synthesis of CDP-diacylglycerol is not a rate-limiting step in the CDP-diacylglycerol pathway [53]. Moreover, phosphatidylserine synthase, a key enzyme in the CDP-diacylglycerol pathway, is inhibited by CTP [20]. Thus, the inhibition of a CDP-diacylglycerol pathway enzyme favors the synthesis of phospholipids by the Kennedy pathway. Indeed, the CDP-diacylglycerol and Kennedy pathways compensate for each other to maintain phospholipid composition [8,54].

Other consequences of the misregulation of CTP synthetase by CTP product inhibition (i.e., E161K mutation) include a 1.6-fold increase in the ratio of neutral lipids to phospholipids [45]. The increase in neutral lipids is due to increased levels of triacylglycerols (1.4-fold), free fatty acids (1.7-fold), and ergosterol esters (1.8-fold) [45]. The mechanism(s) for these changes have not been addressed, but may be related to the increase in phosphatidate concentration that occurs in response to elevated levels of CTP [45]. Recent studies have shown that phosphatidate plays a central role in the transcriptional regulation of lipid synthesis in *S. cerevisiae* [11]. Indeed, cells that express the E161K mutation excrete inositol [45], a characteristic phenotype that typifies the misregulation of phospholipid synthetic genes when cells accumulate an excess of phosphatidate [11]. The increase in phosphatidate might be due to an increase in phospholipase D activity and/or due to activation of another enzyme(s) that generate this phospholipid.

Fig. 3. Domain structure of *URA7*-encoded CTP synthetase. The diagram shows the approximate positions of the CTP synthetase domain and the glutamine amide transfer domain in the CTP synthetase sequence. The sites involved in CTP product inhibition and phosphorylations by protein kinases A and C are indicated.

5. Regulation of CTP synthetase by phosphorylation

Phosphorylation is a posttranslational modification by which various cellular enzymes and transcription factors are regulated [55,56]. Phosphorylation may stimulate or inhibit the function of an enzyme or transcription factor [57,58]. It may also regulate the oligomerization of enzymes and transcription factors and control protein stability [59]. The localization and/or translocation of some enzymes and transcription factors are also controlled by phosphorylation [59–65]. The *S. cerevisiae URA7*-encoded CTP synthetase is phosphorylated by protein kinase A and by protein kinase C. Protein kinase A is cyclic-AMP dependent protein kinase that mediates signals through the Ras-cAMP pathway [66,67]. Protein kinase C is a lipid-dependent protein kinase required for *S. cerevisiae* cell cycle [68–72] and plays a role maintaining cell wall integrity [73]. The phosphorylation of purified *URA7*-encoded CTP synthetase by protein kinase A and protein kinase C results in

the stimulation of enzyme activity by 190% and 300%, respectively [74,75]. *In vivo* labeling studies have shown that the phosphorylation of CTP synthetase by protein kinase A [74] and protein kinase C [75] is physiologically relevant. The stimulation of CTP synthetase by protein kinases A or C may be related to signal transduction pathways that direct the cell growth, proliferation, and biosynthesis of macromolecules. Kinetic analyses show that the mechanisms for stimulation of CTP synthetase activity by the two different protein kinases are the same [74,75]. Phosphorylated CTP synthetase shows an increase in the V_{max} with respect to the substrates UTP and ATP, a decrease in the K_m value toward ATP, and a decrease in the positive cooperativity of the enzyme for ATP [74,75]. Moreover, the phosphorylation of CTP synthetase by protein kinases A and C attenuates the regulation of its activity by CTP product inhibition [74,75].

The effects of phosphorylation on the regulation of *URA7*-encoded CTP synthetase activity involve the oligomerization of the enzyme [22,23]. CTP synthetase exists as a dimer in the absence of its substrates ATP and UTP, but in the presence of saturating concentrations of these substrates the enzyme exists as a tetramer [22,23] (Fig. 4). The kinetics of enzyme tetramerization correlates with the kinetics of enzyme activity. The product CTP does not inhibit the ATP/UTP-dependent tetramerization of the enzyme [22,23]. Phosphorylation of native CTP synthetase with protein kinases A and C facilitates the nucleotide-dependent tetramerization, whereas dephosphorylation of native CTP synthetase with alkaline phosphatase prevents its nucleotide-dependent tetramerization [23]. This correlates with the inactivation of CTP synthetase activity [23]. The rephosphorylation of the enzyme with protein kinases A and C results in a partial restoration of the nucleotide-dependent tetramerization of the enzyme, and this correlates with the partial restoration of CTP synthetase activity [23] (Fig. 4).

A combination of biochemical and molecular approaches has been used to identify protein kinase A and protein kinase C sites in the *URA7*-encoded CTP synthetase [76–78]. Ser^{424} is a target site for both protein kinase A and protein kinase C [76,78]. The phosphorylation of this site is required to maintain optimum CTP synthetase activity *in vivo* [76,78]. Protein kinase C also phosphorylates Ser^{36}, Ser^{330}, Ser^{354}, and Ser^{454} [77]. Biochemical and physiological analyses of Ser-to-Ala mutations have shown that

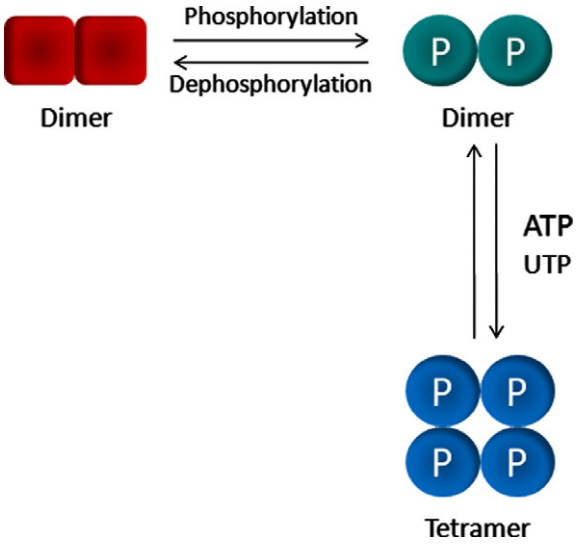

Fig. 4. Model for the role of phosphorylation in the nucleotide-dependent tetramerization and activation of the *URA7*-encoded CTP synthetase. The square represents the dephosphorylated CTP synthetase subunit, and the circle containing the letter P represents the phosphorylated enzyme subunit. The model is adapted from Ref. [23].

phosphorylations at all sites, with the exception of Ser^{330}, result in the stimulation of CTP synthetase activity [77,78]. The phosphorylation of Ser^{330} results in the inhibition of activity [77]. Moreover, *in vivo* studies using these mutants have shown that the regulatory effects of the phosphorylations at specific sites have an impact on the pathways by which membrane phospholipids are synthesized. Phosphorylations at Ser^{36}, Ser^{354}, and Ser^{454} correlate with an increase in PC synthesis via the Kennedy pathway [77,78]. In contrast, phosphorylation at Ser^{330} correlates with a decrease in the utilization of the Kennedy pathway [77].

6. *S. cerevisiae* is a surrogate to study the regulation of human CTP synthetase

The yeast expression system, like bacterial systems, facilitates the isolation of proteins for structure-function studies. However, the yeast expression system has the advantage over bacterial systems in that the effects of posttranslational modifications (e.g., phosphorylation) on human enzymes can be evaluated. The human and *S. cerevisiae* CTP synthetase enzymes have a relatively high degree of amino acid sequence identity (~53%). Indeed, the human CTP synthetase genes (i.e., *CTPS1* and *CTPS2*) are functionally expressed in *S. cerevisiae* [79]. Moreover, the *CTPS1* or *CTPS2* genes rescue the lethal phenotype of the *S. cerevisiae ura7Δ ura8Δ* double mutant that lacks CTP synthetase activity [79].

In vivo labeling experiments with *S. cerevisiae* cells have revealed that the human *CTPS1*-encoded CTP synthetase is phosphorylated [79]. Moreover, the extent of enzyme phosphorylation nearly doubles when yeast cells are activated for the Ras-cAMP pathway and protein kinase A activity [79] and when cells are activated for protein kinase C activity [80]. Indeed, the human CTP synthetase expressed and purified from *S. cerevisiae ura7Δ ura8Δ* cells is phosphorylated by mammalian forms of protein kinase A [81] and protein kinase C [80]. Biochemical and molecular studies have shown that Thr^{455} is a major target site of both protein kinase A and protein kinase C phosphorylation [80,81]. Likewise, Ser^{462} has been identified as a major target site for protein kinase C [80]. Results of *in vitro* and *in vivo* analyses of T455A and S462A mutant CTP synthetase 1 enzymes support the conclusion that phosphorylation at Thr^{455} results in the inhibition of CTP synthetase 1 activity while phosphorylation at Ser^{462} stimulates activity [80,81]. When expressed in *S. cerevisiae*, the T455A mutation in the human enzyme causes a 2.5-fold increase in the cellular concentration of CTP and a 1.5-fold increase in the choline-dependent synthesis of phosphatidylcholine [81]. The effects of the S462A mutation of the human enzyme on phospholipid synthesis in *S. cerevisiae* have not been examined. Protein kinase A and protein kinase C phosphorylate human CTP synthetase 1 on additional serine and threonine residues, but the identification of those sites has not been determined.

Interestingly, the direct effect of protein kinase A phosphorylation on human CTP synthetase 1 activity is opposite to that of the *S. cerevisiae URA7*-encoded CTP synthetase [74,76]. As discussed above, the yeast enzyme is phosphorylated on Ser^{424}, and this phosphorylation results in the stimulation of CTP synthetase activity [76]. The protein kinase A phosphorylation sites in the *S. cerevisiae* (i.e., Ser^{424}) and the human (i.e., Thr^{455}) CTP synthetases are not conserved. The opposing effects of the phosphorylation of these sites could reflect differences in the regulatory functions of protein kinase A in these organisms. In *S. cerevisiae*, the activation of Ras and the subsequent elevation in protein kinase A activity by cAMP production are associated with rapid cell growth and enhanced metabolic activity of the cell [66,67,82]. The stimulation of yeast CTP synthetase activity by protein kinase A phosphorylation might provide a mechanism by which the Ras-cAMP pathway

mediates the synthesis of the CTP required for yeast cell growth. In contrast, cAMP levels and protein kinase A activity are not controlled through the Ras pathway in mammalian cells, and the roles of protein kinase A activity in cell physiology are much more complex than in *S. cerevisiae* [56]. The inhibition of CTP synthetase 1 activity in response to protein kinase A phosphorylation may be a mechanism by which CTP synthesis and cell growth are attenuated in specific tissues/organs in humans. Indeed, the protein kinase A and protein kinase C phosphorylations of CTP synthetase 1 are not detected in human embryonic kidney 293 cells [83]. In this cell type, the major protein kinase that phosphorylates CTP synthetase 1 is glycogen synthase 3, and this phosphorylation inhibits CTP synthetase activity [83].

7. Summary and perspectives

In this review, we have summarized our current understanding of CTP synthetase in *S. cerevisiae*. The *URA7*-encoded CTP synthetase is responsible for the majority of CTP synthesized *in vivo*, and the enzyme is regulated on a biochemical level by CTP product inhibition and by phosphorylations via protein kinases A and C. These forms of regulation govern the cellular levels of CTP, which in turn influence the utilization of the Kennedy and CDP-diacylglycerol pathways of phospholipid synthesis. The *URA8*-encoded enzyme is also regulated by CTP product inhibition and contains putative phosphorylation sites. We speculate that phosphorylation also regulates the *URA8*-encoded enzyme, and that this modification has effects on cellular functions that depend on CTP.

Studies with the yeast CTP synthetases have spurred studies on the human forms of the enzyme. The expression of human CTP synthetase enzymes in a yeast mutant that lacks CTP synthetase has facilitated structure-function analysis of protein kinase A and protein kinase C target sites. While the CTP synthetase enzymes from yeast and human cells are similar, there are differences with respect to the sites of phosphorylation, and the effects of phosphorylation on CTP synthetase activity. Comparison of their structures might shed light on the molecular basis for these differences.

Aside from being an essential precursor for membrane phospholipid synthesis, CTP is a precursor for the synthesis of RNA, DNA, and sialoglycoproteins [55,84,85]. In mammalian systems, CTP synthetase mutations that alleviate CTP product inhibition result in abnormally high intracellular levels of CTP and dCTP [45,86,87], resistance to nucleotide analog drugs used in cancer chemotherapy [88–91], and increased rates of spontaneous mutations [48,89,91]. As discussed above, CTP synthetase activity controls the balance of nucleotide pools [4,5,22,45,48,86,92] and regulates the pathways by which phospholipids are synthesized [20,45,93]. The importance of understanding the regulation of CTP synthetase is further emphasized by the fact that elevated/unregulated CTP levels are characteristic of many forms of cancer [48,86,93–103]. Accordingly, CTP synthetase is a target for cancer therapy. In addition, the enzyme is a target for treating human diseases (e.g., African sleeping sickness [27,104], malaria [105,106], and blindness [107]) that are caused by parasites. CTP synthetase inhibitors such as cyclopentenylcytosine [108,109] and 3-deazauridine [110,111] that are currently available are not entirely specific. Based on the importance of phosphorylation in the regulation of CTP synthetase activity, novel inhibitors may be discovered and/or designed that are based on key phosphorylation sites in the enzyme. There are still numerous phosphorylations sites in the human CTP synthetase enzymes that have yet to be characterized, and clearly, additional studies toward this end are warranted. The expression of human CTP synthetase enzymes in *S. cerevisiae* would facilitate the evaluation of inhibitors that control cellular CTP levels.

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