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Expression in *Escherichia coli* of the *Saccharomyces cerevisiae CCT* Gene Encoding Cholinephosphate Cytidylyltransferase

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The coding region of the *CCT* gene from the yeast *Saccharomyces cerevisiae* was cloned into the pUC18 expression vector. The plasmid directed the synthesis of an active cholinephosphate cytidylyltransferase in *Escherichia coli*, confirming that *CCT* is the structural gene for this enzyme. The enzyme produced in *E. coli* efficiently utilized cholinephosphate and *N*,*N*-dimethylethanolaminephosphate, but *N*-methylethanolaminephosphate and ethanolaminephosphate were poor substrates. Consistently, disruption of the *CCT* locus in the wild-type yeast cells resulted in a drastic decrease in activities with respect to the former two substrates. When activity was expressed in *E. coli*, over 90% was recovered in the cytosol, whereas most of the activity of yeast cells was associated with membranes, suggesting that yeast cells possess a mechanism that promotes membrane association of cytidylyltransferase.

CTP:cholinephosphate cytidylyltransferase (EC 2.7.7.15; hereafter referred to as CCTase) catalyzes the conversion of cholinephosphate into CDP-choline. This enzyme has been the subject of recent intensive studies because it has been shown to play a major role in the regulation of phosphatidylcholine synthesis in mammalian tissues (10). The control mechanism involves translocation of the enzyme from the cytosol to membranes, where the enzyme is activated through interaction with phospholipids. This translocation was shown to be promoted by a number of agents known to stimulate phosphatidylcholine synthesis, such as free fatty acid (9), 12-*O*-tetradecanoyl phorbol 13-acetate (8), phospholipase C treatment (15), and choline deficiency (21).

Although such regulation has not yet been demonstrated for the yeast (*Saccharomyces cerevisiae*) enzyme, we have been attracted to the function and role of this enzyme in yeast cells. For this reason, we previously isolated a yeast mutant carrying a thermolabile CCTase (7) and used it to clone a yeast DNA fragment that could complement the mutation (19). Introduction of the cloned DNA on a multicopy plasmid allowed a high level of CCTase expression in yeast cells. The enzyme produced by the transformant was indistinguishable from that found in the wild-type yeast strain. An open reading frame capable of encoding a protein with a relative molecular weight of 49,379 was found in the cloned DNA fragment. We called this gene *CCT* and inferred that it is the structural gene for CCTase. To confirm and extend this view, we attempted to express the *CCT* gene in *Escherichia coli* cells that were originally deficient in this enzyme.

The *Dra*I fragment of *CCT* encompassing nucleotide positions 600 to 1895 (amino acids 25 to the C terminus) (19) was inserted into the *Sma*I site of the pUC18 vector shortly after the β-galactosidase start codon (20). As a result, the N-terminal 24 amino acids of the original CCTase sequence were replaced with the N-terminal 11 amino acids from *E. coli* β-galactosidase (Fig. 1). The resulting plasmid, pUC-CCT, was used for lactose-inducible expression of CCTase in *E. coli*. pUC-CCTO, containing the aforementioned *Dra*I fragment in the opposite orientation, was also constructed and used as a control. *E. coli* JM103 [Δ(*lac-pro*) *thi strA endA sbcB15 hsdR4 supE* (F′ *traD36 proAB lacI*^{q} *lacZ M15*)] was transformed with plasmid pUC-CCT or pUC-CCTO and cultured in LB medium in the presence of ampicillin and isopropylthio-β-D-galactopyranoside, and then a cell extract was prepared as described previously (6). Its CCTase activity was determined essentially as described previously (7), with some modifications. Briefly, the complete assay system contained 50 mM Tris hydrochloride (pH 8.0), 5 mM CTP, 25 mM $MgCl_2$, 0.1 mM [*methyl*-^{14}C]cholinephosphate (13,000 cpm/nmol), and the cell extract in a total volume of 20 μl and was incubated at 30°C for 30 min. The reaction product was separated by thin-layer chromatography on a silica gel 60 plate with ethanol–2% ammonia (1:1, vol/vol) and located by autoradiography, and then its radioactivity was counted. The *E. coli* transformant harboring pUC-CCT contained significant CCTase activity (Fig. 2, lane 2). The specific activity was estimated to be 12.6 μmol g^{-1} min^{-1}, several times higher than that of the wild-type yeast cells but lower than that of yeast transformants carrying multiple copies of *CCT*. The activity was stimulated by Mg^{2+} (lane 3) and was dependent on CTP (lane 4). When an extract prepared from the pUC-CCTO-harboring cells was used as the enzyme source, activity was undetectable (lane 5). Thus, *CCT* directed the synthesis of an active CCTase in *E. coli*, showing that *CCT* can encode the entire CCTase sequence. This result supports our notion that the *CCT* gene is the structural gene for CCTase (19). The rat liver and pea enzymes were also reported to comprise single species of polypeptide chains with M_r values of 45,000 (2) or 42,000 (13) and 56,000 (11), respectively.

In addition to CCTase, yeast cells possess ethanolaminephosphate cytidylyltransferase (EC 2.7.7.14) (5, 17). This enzyme was shown to be distinct from CCTase in yeast cells (7) as well as in mammalian tissues (14), but the substrate specificities of the respective cytidylyltransferases have not yet been precisely determined. In our previous study (7), we showed that a yeast mutant (*cct pss*) defective in both CCTase and phosphatidylserine synthase could grow on ethanolamine or *N*-methylethanolamine but not on *N*,*N*-dimethylethanolamine or choline. This finding strongly sug-

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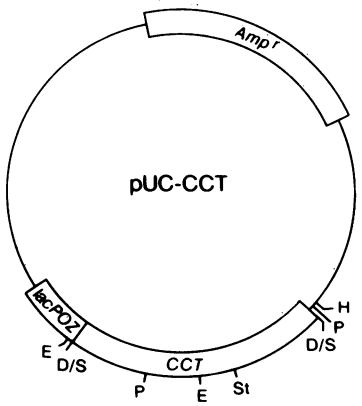
A



B

pUC18 *lacZ* 5'AGGAAACAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCGGGGATC...
M T M I T N S S S V P G D

pUC-CCT *CCT* 5'AGGAAACAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCAAAAAA...
M T M I T N S S S V P K K

yeast *CCT* 5'GAAAAATGGCAAACCCAACAACAGGG...CTATCAAACCTATTTAAAAAA...
M A N P T T G L S N L F K K

FIG. 1. Plasmid pUC-CCT, used for the expression of yeast CCTase in *E. coli*. (A) Structure of the plasmid. Abbreviations for restriction enzyme sites: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; St, *Stu*I; D/S, junction of the *Dra*I and *Sma*I sites. (B) Amino-terminal sequences of pUC18 *lacZ*, pUC-CCT *CCT*, and yeast *CCT*. The *E. coli* ribosome-binding site is indicated by the broken lines. The *Sma*I and *Dra*I sites used for the construction of pUC-CCT are marked with the straight and wavy underscores, respectively.

gests that yeast CCTase utilizes *N*,*N*-dimethylethanolaminephosphate as well as cholinephosphate as a substrate, but *N*-methylethanolaminephosphate and ethanolaminephosphate were poorly utilized as substrates. To confirm this conclusion, we used the extract from the CCTase-expressing *E. coli* since *E. coli* is deficient in ethanolaminephosphate cytidylyltransferase activity. ^{32}P-Labeled cholinephosphate analogs with identical specific radioactivities were enzymatically prepared by phosphorylation of the corresponding choline analogs with [γ-^{32}P]ATP. *N*-Methylethanolaminephosphate and *N*,*N*-dimethylethanolaminephosphate were prepared by the method of Ansell and Chojnacki (1). Ethanolaminephosphate and cholinephosphate were prepared as described by Sundler (18) and Hosaka et al. (3), respectively. Because of their limited supply, the ^{32}P-labeled substrates were used at a reduced level. A 50-μg sample of the extract from *E. coli* cells carrying pUC-CCT was incubated at 30°C for 60 min with the aforementioned CCTase assay mixture containing one of these ^{32}P-labeled substrates (4×10^3 cpm; approximately 0.5 nM) instead of 0.1 mM [*methyl*-^{14}C]cholinephosphate. The reaction product was separated by thin-layer chromatography and subjected to autoradiography, after which the radioactivity in the product was counted. The relative activities toward cholinephosphate, *N*,*N*-dimethylethanolaminephosphate, *N*-methylethanolaminephosphate, and ethanolaminephosphate were 100, 66, 11, and 2, respectively. Thus, cholinephosphate was the best substrate for the enzyme and *N*,*N*-dimethylethanolaminephosphate was also a good substrate, but *N*-methylethanolaminephosphate and ethanolaminephosphate were poor substrates.

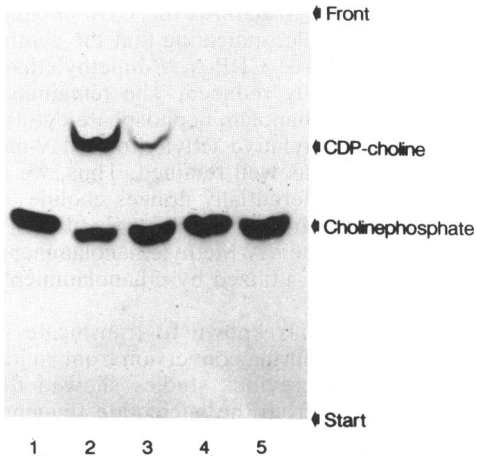


FIG. 2. Thin-layer chromatography of the reaction product. An extract was prepared from *E. coli* cells harboring the CCTase-expressing vector pUC-CCT, and 50 μg of protein was incubated with the CCTase assay mixture as indicated. The reaction product was separated by thin-layer chromatography and located by autoradiography. Lanes: 1, minus cell extract; 2, complete system; 3, minus $MgCl_2$; 4, minus CTP. In lane 5, an extract from *E. coli* cells harboring pUC-CCTO instead of pUC-CCT was used.

To confirm this finding, the *CCT* locus of the wild-type yeast genome was disrupted and the loss of cytidylyltransferase activities was analyzed. The 1.6-kb *Hin*dIII fragment of the *CCT* gene (19) was transferred to pUC19. The resulting plasmid was digested with *Bal*I and *Hpa*I, and the 0.9-kb sequence containing more than two-thirds of the *CCT* coding region was removed and filled in with the 1.1-kb Klenow-treated *Hin*dIII fragment carrying the yeast *URA3* gene (12). Plasmid pUC-cct::URA3 thus obtained was linearized with *Hin*dIII and used for transformation of haploid yeast strain D417-1 (**a** *leu2 his3 ura3 trp1 ade8*) by the lithium acetate method of Ito et al. (4). Ura$^+$ colonies were selected, and their gene disruption was confirmed by Southern blot analysis (16) (data not shown). The homogenate prepared from the *cct* disruptant (strain 2019) was used for the determination of different cytidylyltransferase activities. Table 1 shows the activities of wild-type strain X2180-1B (*SUC2 mal gal2 CUP1*) and *cct* disruptant 2019. The wild-type cells mediated the cytidylylation of cholinephosphate and ethanolaminephosphate at comparable rates. The activity toward *N*,*N*-dimethylethanolaminephosphate was somewhat higher than that expected from the value obtained with the *E. coli*-expressed enzyme (see above). Probably ethanolaminephosphate cytidylyltransferase had some activity toward this

TABLE 1. Cytidylyltransferase activities in the wild type and *cct* disruptant

Substrate	Relative activity[a]	
	Wild type	*cct* disruptant
N-Methylethanolaminephosphate	41	34
N,*N*-Dimethylethanolaminephosphate	94	19
Chlinephosphate	109	3

[a] Relative to the value obtained with ethanolaminephosphate as the substrate (set at 100). Activities of the wild type and disruptant for ethanolaminephosphate were 3.0×10^5 and 1.9×10^5 cpm g^{-1} min^{-1}, respectively.

substrate. In disruptant 2019, however, CDP-choline synthesis was hardly detectable, indicating that the synthesis was entirely due to CCTase. CDP-*N*,*N*-dimethylethanolamine synthesis was drastically reduced. The remaining activity was probably due to ethanolaminephosphate cytidylyltransferase. In contrast, the relative activity toward *N*-methylethanolaminephosphate was well retained. Thus, we conclude that yeast CCTase preferentially utilizes choline and *N*,*N*-dimethylethanolaminephosphate but poorly utilizes *N*-methylethanolaminephosphate. *N*-Methylethanolaminephosphate is thought to be mainly utilized by ethanolaminephosphate cytidylyltransferase.

Mammalian CCTase is known to translocate from the cytosol to membranes during conversion from an inactive to an active form. Our previous studies showed that yeast CCTase, as predicted from the nucleotide sequence of the encoding gene, is a rather hydrophilic protein without extensive hydrophobic stretches (19). We were therefore interested in determining the subcellular localization of the enzyme in the *E. coli* transformant as well as in yeast cells. Homogenates were prepared from the *E. coli* transformant and the wild-type yeast strain (X2180-1B) and fractionated into the supernatant and particulate fractions by centrifugation at 100,000 × *g* for 60 min. The *E. coli* transformant contained 90.7% of the CCTase activity in the cytosol, supporting the notion that the CCTase is by itself a soluble protein. However, yeast cells contained 93.5% of the CCTase activity in the membrane fraction. When the enzyme was overexpressed in yeast cells by using multiple copies of *CCT*, the activity was still largely recovered in the membrane fraction (89 to 92%). These results strongly suggest that the yeast possesses a mechanism promoting the association of CCTase with the cell membrane. The failure of *E. coli* cells to bind CCTase to their membranes supports the view that *E. coli* lacking CCTase does not have this mechanism. It is unlikely that this failure was due to the modification of the N-terminal sequence of CCTase. In vitro modification and expression in yeast cells of the *CCT* gene will provide an effective method for elucidating the mechanism for membrane binding of CCTase in *S. cerevisiae*.

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