IDENTIFICATION OF IMPORTANT RESIDUES FOR CATALYSIS AND SUBSTRATE SPECIFICITY IN HUMAN CHOLINE KINASE BY SITE DIRECTED MUTAGENESIS

by

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March 2008

CERTIFICATE

This is to certify that the dissertation entitled

Identification of important residues for catalysis and substrate specificity in human choline kinase by site directed mutagenesis

is the bonafide record of the research work done by

Wong Mun Teng

during the period from July 2007 to March 2008

under my supervision.

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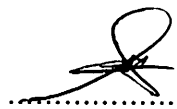

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

APH	Aminoglycoside phosphotransferase
APS	Ammonium persulphate
bp	Base pair
BSA	Bovine serum albumin
CK	Choline kinase
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EK	Ethanolamine kinase
GST	Glutathione sulfur transferase
HC-3	Hemicholinium-3
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	Kilo Dalton
LB	Luria-Bertani
LDH	Lactate dehydrogenase
NADH	Nicotinamide adenine dinucleotide
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PK	Pyruvate kinase

SDS	Sodium dodecyl sulphate
TEMED	N, N, N', N' Tetramethyl-ethylenediamine
Tris	Tris(hydroxymethyl)-aminomethane
T_m	Melting temperature
U	Unit
UV	Ultraviolet
w/v	Weight per volume
v/v	Volume per volume

PENGENALPASTIAN RESIDU PENTING UNTUK PEMANGKINAN DAN SPESIFIKASI SUBSTRAT DALAM KOLINA KINASE MANUSIA DENGAN MUTAGENESIS

ABSTRAK

Fosfatidilkolina (PtdCho) merupakan komponen penting untuk membran eukaryotik dan sebahagian membran prokaryotik. Kolina kinase (CK) ialah enzim pertama dalam laluan CDP-kolina. CK menukarkan kolina kepada fosforilkolina. CK dijumpai di dalam bahagian supernatan sel. CK telah dikenali sebagai satu sasaran baru untuk terapi anti-kanser. Untuk mengenalpasti residu asid amino CK alfa 2 manusia (hCKα2) yang penting untuk proses pemangkinan, aspartat di posisi 342 telah dimutasi kepada asparagina. Binaan mutan telah berjaya diklonkan ke dalam vektor pET14b dan lebihan diekspresi dalam *E.coli* BL21(DE3). Protein mutan D342NhCKα2 telah menunjukkan kehilangan aktiviti dramatik, hanya 12.46% daripada aktiviti protein asli yang tinggal. Protein mutan menunjukkan peningkatan nilai K_m sebanyak 1.18 kali untuk kolina, manakala peningkatan nilai K_m untuk etanolamina adalah 598 kali berbanding dengan protein asli. Nilai K_m dan V_{max} untuk ATP bagi protein mutan meningkat 3 dan 4 kali masing-masing. Peningkatan nilai K_m mencadangkan bahawa residu ini penting untuk pelekatan substrat dan memainkan peranan dalam proses pemangkinan. Mutasi pada aspartat 342 mungkin menyebabkan perencatan aktiviti atau gangguan bagi kompleks homo-dimer dalam hCKα2.

IDENTIFICATION OF IMPORTANT RESIDUES FOR CATALYSIS AND SUBSTRATE SPECIFICITY IN HUMAN CHOLINE KINASE BY SITE-DIRECTED MUTAGENESIS

ABSTRACT

Phosphatidylcholine (PtdCho) is a prominent constituent of eukaryotic and some prokaryotic membranes. Choline kinase (CK), the initial enzyme of the CDP-choline pathway, mediates the conversion of choline to phosphorylcholine and is localized in the supernatant fraction of cells. CK has been recognized as a new target for anticancer therapy. To identify the amino acid residue of human CK (hCK) that is important for catalysis, conserved aspartate at position 342 in hCKα2 was mutated to asparagine. The mutant construct was successfully cloned into pET14b vector and overexpressed in *E.coli* BL21(DE3). The mutant protein D342NhCKα2 showed dramatic loss of activity, only 12.46% of wild type protein activity remained. The K_m for choline of the mutant protein increased 1.18 folds while K_m for ethanolamine increased 598 folds compared to wild type. The K_m and V_{max} for ATP of mutant protein increased 3 and 4 folds respectively. The increased K_m suggested that the residue is important for the binding of the substrates and play a role in catalysis. Mutation of aspartate 342 might also cause the activity inhibition or disruption of homo-dimer complex in hCKα2.

CHAPTER 1.0 INTRODUCTION

Phospholipid plays many important roles in nature. Their best known role is to form membrane bilayers, critical components of cells of all earthly life form. The phospholipids bilayer that surrounds mammalian cells consists of four major phospholipids components: phosphatidylcholine (PtdCho), sphingomyelin, phosphatidylethanolamine and phosphatidylserine. The choline containing lipids (phosphatidylcholine and sphingomyelin) are predominantly (60-80%) in the extracellular leaflet, whereas the aminophospholipids (phosphatidylethanolamine and phosphatidylserine) are predominantly (60-80%) in the inner membrane leaflet, although this distinction does vary with tissue types (Sher et al, 2006). Phosphatidylcholine and phosphatidylethanolamine represent 44% and 18% of total phospholipids in yeast, respectively. In addition these two phospholipids represent 40-50% and 35-40% of the total phospholipids in *Plasmodium falciparum* (Pessi et al, 2004).

1.1 Phosphatidylcholine

Phophatidylcholine (PtdCho) is the most abundant class of phospholipids in eukaryotic cells. Phosphatidylcholine also present in select prokaryotes including *Treponema denticola*, whose genome encodes a fusion protein containing choline kinase and CTP:phosphocholine cytidylyltrasnferase activity (Kent et al, 2004). Phosphatidylcholine is comprised of hydrocarbon chains attached to

glycerophosphocholine through acyl, alkyl or alkenyl linkage (Figure 1). It is a neutral or zwitterionic phospholipid over a pH range from strongly acidic to strongly alkaline.

1.1.1 Biosynthesis of phosphatidylcholine

The biosynthesis of phosphatidylcholine is accomplished by three distinct pathways. In eukaryotic organisms, phosphatidylcholine can be synthesized by two alternative biosynthesis pathways, CDP-choline pathway or the methylation pathway (Figure 2) (Lopez-Lara & Geiger, 2001). Many prokaryotes lack of phosphatidylcholine but it can be found in significant amounts in membrane of distantly related bacteria such as Rhizobacteria and Spirochetes. Enzymatic methylation of phosphatidylethanolamine via methylation pathway was thought to be the only biosynthesis pathway to yield phosphatidylcholine in bacteria. However, a choline dependent pathway for phosphatidylcholine biosynthesis has been discovered in *Sinorhizobium meliloti* (Figure 3) (Sohlenkamp et al, 2003).

CDP-choline pathway is also known as Kennedy pathway. The first step of the pathway is the phosphorylation of choline to phosphorylcholine (PChol). Choline kinase (CK) catalyzes this phosphorylation by ATP in the presence of magnesium ion (Mg^{2+}), yielding phosphorylcholine and ADP. CTP:phosphocholine cytidylyltransferase (CCT) catalyzes the formation of CDP-choline (CDP-Cho) from phosphorylcholine and CTP. Cholinephosphotransferase (CPT) catalyzes the final condensation reaction of CDP-choline with 1,2-diacylglycerol to form phosphatidylcholine (Kent, 2005).

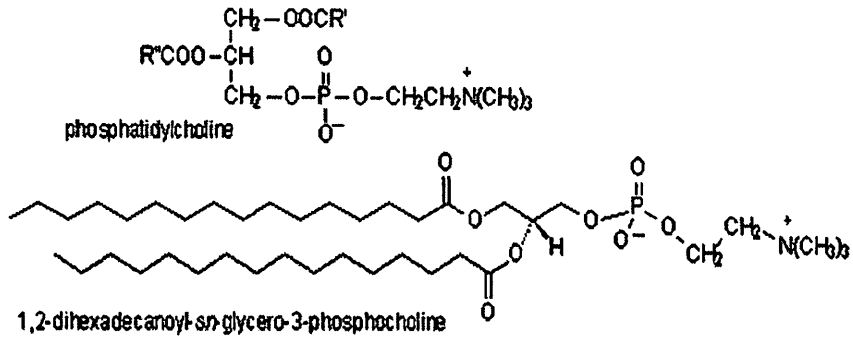

Figure 1: Formula of phosphatidylcholine (www.lipidlibrary.co.uk). Phosphatidylcholine consists of hydrocarbon chains bind to the glycerophosphocholine through acyl, alkyl or alkenyl linkage. R' and R" represent the alkyl parts of fatty acyl residues.

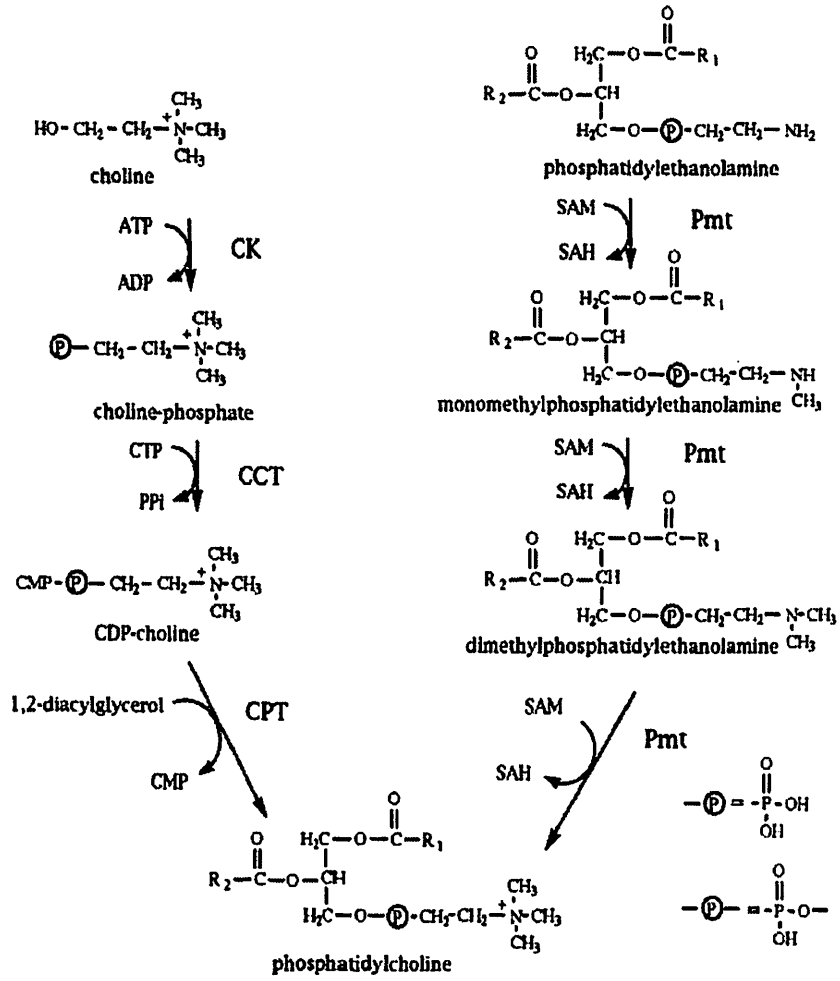

Figure 2: Phosphatidylcholine biosynthesis in eukaryotes (Lopez-Lara & Geiger, 2001). Phosphatidylcholine is synthesized by two pathways: CDP-choline pathway (left) or methylation pathway (right). Choline kinase (CK), CTP: phosphocholine cytidylyltransferase (CCT) and choline phosphotransferase (CPT) catalyse the reactions in the CDP-choline pathway. While phosphatidylethanolamine methyltransferase (Pmt) catalyses the three steps methylation of phosphatidylethanolamine in the methylation pathway. SAM, S-adenosylmethionine act as the donor of methyl group; SAH, S-adenosylhomocysteine; R1 and R2 represent the alkyl parts of fatty acyl residues.

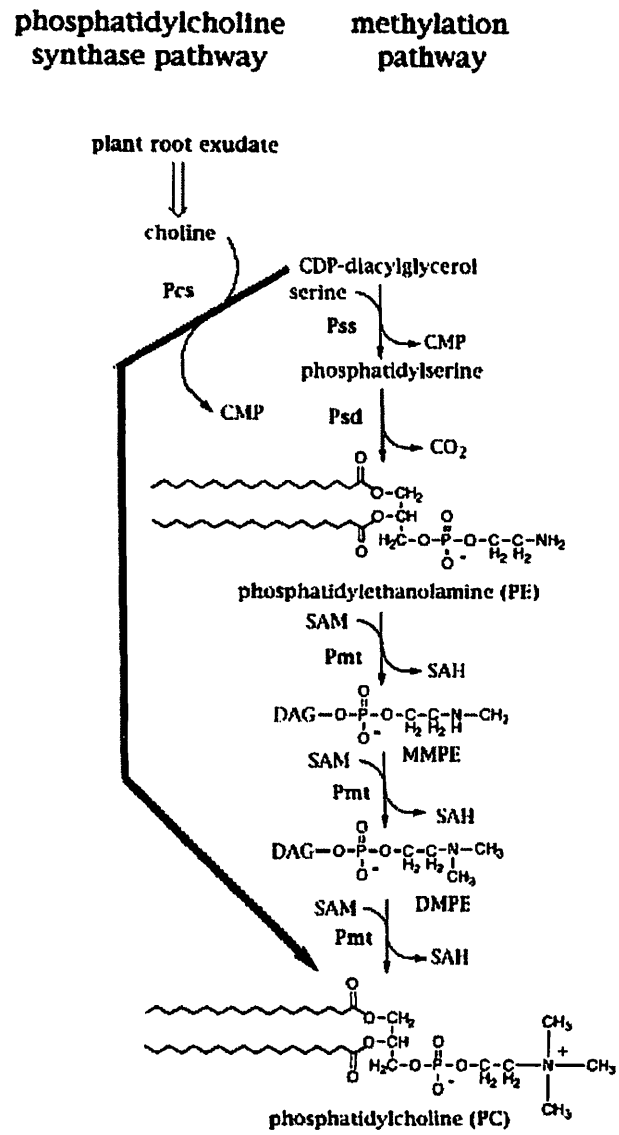

Figure 3: Phosphatidylcholine biosynthesis in the Sinorhizobium meliloti (taken from Sohlenkamp et al, 2003), the third phosphatidylcholine biosynthesis pathway (➟). Choline from the plant root exudates reacts with CDP-diacylglycerol to form phoshatidylcholine and CMP. Phosphatidylcholine synthase (Pcs) involve in this enzymatic activity. Psd: phosphatidylserine decarboxylase; SAM: S-adenosylmethionine; SAH: S-adenosyl homocysteine. Beside methylation pathway for the phosphatidylcholine synthesis also shows.

Phosphatidylcholine molecules produced from the CDP-choline pathway were mainly comprised of medium chain, saturated fatty acid species (DeLong et al, 1999).

The second pathway for the phosphatidylcholine biosynthesis involves sequential methylation of phosphatidylethanolamine, with S-adenosylmethionine (SAM) as the source of methyl groups (Figure 2). Phosphatidylethanolamine methyltransferase catalyzed the first methylation process in this pathway to produce monomethylphosphatidylethanolamine (MMPE). While the second and third methylations are catalyzed by phospholipids methyltransferase yielding dimethylphosphatidylethanolamine (DMPE) and phosphatidylcholine respectively (Kodaki & Yamashita, 1987). Although methylation pathway is a minor pathway for the higher organisms, it becomes the main route to phosphatidylcholine in most bacteria species, yeasts and mammalian hepatocytes (DeLong et al, 1999). In addition, phosphatidylcholine molecules from the phosphatidylethanolamine methylation pathway were much more diverse and comprised of significantly more long chain, polyunsaturated species. Phosphatidylcholine species from the methylation pathway contained a higher percentage of arachidonate and are more diverse than those from CDP-choline pathway (DeLong et al, 1999).

The third pathway, so far found in certain bacteria, involves the reaction of choline with CDP-diacylglycerol to form phosphatidylcholine and CMP (Figure 3).The pathway for biosynthesis in *S. meliloti* involves phosphatidylcholine synthase (Pcs) to form

phosphatidylcholine in one step (Kent, 2005). A number of symbiotic (*Rhizobium leguminosarum, Mesorhizobium loti*) and pathogenic (*Agrobacterium tumefaciens*) bacteria seem to posses the phosphatidylcholine synthase pathway and suggest that the eukaryotic host functions as the provider of choline for this pathway (Sohlenkamp et al, 2003).

1.1.2 Functions of phosphatidylcholine

Several recent discoveries revealed that the importance of phosphatidylcholine in the mammalian cell physiology. Alterations of phosphatidylcholine metabolism are associated to different key cellular events such as oncogenic transformation and programmed cell death. Perturbation of phosphatidylcholine synthesis can lead to inhibition of growth or even cell death. While enhanced synthesis of phosphatidylcholine appears to occur in cancer cells and solid tumours. This may provide a target for therapeutic agents (Cui & Houweling, 2002).

Together with other phospholipids like phosphatidylethanolamine and lipids, phosphatidylcholine become the major structural component of the eukaryotic cellular membrane (Exton, 2000). Because of the general cylindrical shape of the molecule, phosphatidylcholine spontaneously organizes into bilayers. So it is ideally suited to serve as the bulk structural element of biological membranes (Culis & Kruijff, 1979). Phosphatidylcholine is a key player in balancing the proportions of bilayer and non-bilayer lipids that determine membrane intrinsic curvature. This balance has been

recognized as a novel criterion in the regulation of yeast membrane lipid composition (de Kroon, 2007).

In addition, phosphatidylcholine also has a role in signaling transduction as a source of lipid signaling molecules. It serves as precursor for the production of lipid second messengers, like phosphatidic acid, lysophosphatidylcholine and platelet activating factor, each with important signaling functions (Exton, 2000). Beside, phosphatidylcholine is the biosynthetic precursor of sphingomyelin and as such must have influence on the many metabolic pathways that constitute the sphingomyelin cycle.

Recently phosphatidylcholine has been suggested to be involved in specific lipid-protein interaction with the yeast mitochondrial glycerol-3-phosphate dehydrogenase (Gut2) based on the result of a photolabeling approach (Janssen et al, 2002).

1.2 Phosphatidylethanolamine

Phosphatidylethanolamine is one of the major phospholipids constituents in biomembranes, second only to phosphatidylcholine. Phosphatidylethanolamine is mainly found in the cytoplasmic leaflet, while phosphatidylcholine is mainly found in the extracellular leaflet. It also serves as precursor for phosphatidylserine, synthesized by a base exchange enzyme, and phophatidylcholine, synthesized by methylation

enzymes (Uchida, 1997).

1.2.1 Biosynthesis of phosphatidylethanolamine

Phosphatidylethanolamine is mainly synthesized through two pathways: CDP-ethanolamine pathway (Figure 4) and decarboxylation pathway (Figure 5). The CDP-ethanolamine pathway is analogous to the CDP-choline pathway. Ethanolamine that is transported into the cytoplasm is phosphorylated by ethanolamine kinase into phosphoethanolamine. The second step in CDP-ethanolamine pathway involves the formation of CDP-ethanolamine and phosphate from CTP and phosphoethanolamine, a reaction catalyzed by CTP:phosphoethanolamine cytidylyltransferase. Finally, the last step involved transfer of phosphoethanolamine from CTP: phosphoethanolamine to diacylglycerol resulting in the formation of phosphatidylethanolamine and CMP. The enzyme catalyzing this reaction is ethanolamine phosphotransferase. CDP-ethanolamine pathway is considered as a major route for phosphatidylethanolamine synthesis in most mammalian tissues (Kent, 1995).

While in the decarboxylation pathway, the most important is the conversion of phosphatidylserine to phsphatidylethanolamine. Phosphatidylserine is synthesized by base exchange reaction with phosphatidylcholine. Then the phosphatidylserine is decarboxylated to phosphatidylethanolamine by phosphatidylserine decarboxylase (Uchida, 1997). In prokaryotic cells like *E. coli* phosphatidylethanolamine is the most abundant membrane phospholipids and all of it is derived from this pathway.

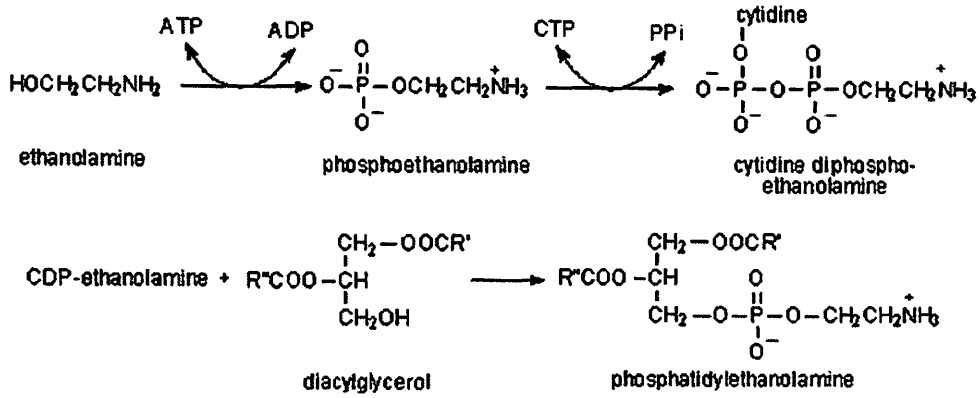

Figure 4: CDP:ethanolamine pathway. Ethanolamine is that transported into the cytoplasm is phosphorylated by ethanolamine kinase, activated with CTP and finally phosphoethanolamine transferase catalyses the reaction of the cytidine diphosphoethanolamine to form phosphotidylethanolamine.

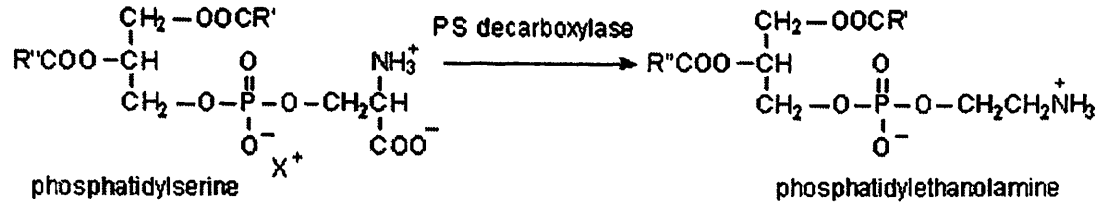

Figure 5: Formation of phosphatidylethanolamine by decarboxylation of phosphatidylserine. Phosphatidylserine is decarboxylated to phosphatidylethanolamine by phosphatidylserine decarboxylase. The decarboxylation takes place in the mitochondria inner membrane.