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Kinetic characterization of mammalian ceramide synthases: Determination of K_m values towards sphinganine

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Abstract **Ceramide is a key metabolite in the pathway of sphingolipid biosynthesis. In mammals, ceramide is synthesized by *N*-acylation of a sphingoid long-chain base by a family of ceramide synthases (CerS), each of which displays a high specificity towards acyl CoAs of different chain lengths. We now optimize a previously-described assay for measuring CerS activity for use upon over-expression of mammalian CerS, and using these conditions, establish the K_m value of each CerS towards sphinganine. Remarkably, the K_m values towards sphinganine are all similar, ranging from 2 to 5 μM, even for CerS proteins that are able to use more than one acyl CoA for ceramide synthesis (i.e. CerS4). The availability of this assay will permit further accurate characterization of the kinetic parameters of mammalian CerS proteins.**

Keywords: Lipid; Sphingolipid; Ceramide; Acyl CoA; Sphinganine; Assay

1. Introduction

Ceramide is both an intermediate in the biosynthetic pathway of sphingolipids and an important bioactive lipid involved in a number of molecular signalling pathways [1–4]. Ceramide, which consists of a sphingoid long-chain base to which a fatty acid is *N*-acylated, is synthesized by ceramide synthase (CerS). This enzyme was first assayed biochemically in 1966 [5], and since then a number of attempts have been made to optimize assay conditions for CerS ([6–9], Table 1). As with many membrane-bound enzymes, the assay is complicated by the hydrophobic nature of the two substrates, namely acyl CoAs, and sphingoid long-chain bases such as sphinganine or sphingosine.

Recently, CerS were identified molecularly. The first CerS to be characterized was the yeast enzyme, Lag1/Lac1 [10,11], which lead to the subsequent discovery of six mammalian genes, originally named longevity assurance gene homologs (LASS) genes, but recently renamed CerS [12]. Remarkably, each one of the six mammalian CerS proteins synthesizes ceramide with a unique fatty acid composition. For instance, CerS1 synthesizes C18-ceramide [13], CerS5 synthesizes C16-ceramide [14], while CerS2 synthesizes ceramide with longer acyl chains [16 and Laviad et al., unpublished observations]. Thus, assay of each CerS requires the use of different acyl CoAs in the reaction mixture.

We now demonstrate that despite the high selectivity towards acyl CoAs, mammalian CerS have a very similar K_m value towards sphinganine, strengthening the notion that the main biochemical difference between CerS is in their specificity towards acyl CoAs. While obtaining this data, we optimized conditions for assaying CerS activity in cells over-expressing CerS, using a modified detergent-free assay which we previously described to measure CerS activity in rat liver microsomes [7,8], before the molecular identity of CerS had been elucidated. The lack of detergent is important as some detergents disrupt the activity of membrane-bound enzymes (see for instance, [17]), including CerS [11]. Using this modified assay, we demonstrate a K_m value of all six mammalian CerS towards sphinganine in the low μM range (2–5 μM), significantly lower than that suggested using a variety of other assays and tissue sources (reviewed in [9]).

2. Materials and methods

2.1. Expression plasmids

Mouse CerS1 and CerS5 were previously cloned in our laboratory [13,14]. Human CerS3 was sub-cloned from cDNA of keratinocytes (kindly provided by Dr. Walt Holleran, University of California at San Francisco) using *Pfu* polymerase with the following forward and reverse primers, respectively, having unique restriction sites: 5′-CGCGGATCCATGTTTTGGACGTTTAAAG (EcoRI) and 5′-CCGGAATTCCATTGGGAATGAGGTGCCT (HindIII). The PCR products were further sub-cloned into pcDNA3-HA vectors. Human CerS1, 2, 4, 5, and 6 genes were cloned into a pCMV-Tag2B plasmid vector (Stratagene), which contains an N-terminal FLAG tag, as follows: CerS1, kindly provided by S. Michal Jazwinski (Louisiana State University, New Orleans, Louisiana), CerS2 and 5 were cloned using a human liver tissue library (Clontech), CerS4 cDNA clones were purchased from ATCC (catalogue number MGC-26850), and CerS6 was cloned using a human kidney tissue library (Clontech). Genes were inserted into pCMV-Tag2B using the following primers flanked with BamHI (or HinDIII for Cers2) and EcoRI sites: CerS1 (gi18490662), 5′-CGGGATCCGCGGCGGCGGGGCCC, 5′-CGGAATTCGGGGTTCAGAAGCGC; CerS2 (gi33876084), 5′-CCGAATTCCTCCAGACCTTGTATGATTAC, 5′-CGAAGCTTGGGAGCGGGGTAGTTCCTTGGC; CerS4 (gi33988505), 5′-CCGGATCCCTGTCCAGTTTCAACGAG, 5′-GGGAATTCGGCTATGTGGCTGTTGTG; CerS5 (gi21618501), 5′-AAAGGATCCGCGACAGCAGCACAGGGACC, 5′-AAAGAATTCCCCTATAGCAACCACCTTACTC; CerS6

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Table 1
Conditions used for assaying CerS activity in vitro

Tissue source	Buffer	Cation	Detergent	Sphingoid base and acyl CoA	Original Refs.	Other Refs.
Liver microsomes	Tris	None	Tween 20	Sphingosine (coated on Celite)/ C16- and C24:1-CoAs	[5]	
Brain microsomes	Potassium phosphate	Mg^{2+}	Tween 20	Sphingosine (coated on Celite)/ C16-, C18-, and C18:1-CoAs	[6]	[25]
Liver microsomes	Potassium phosphate	None	None	Sphingosine/C16-CoA	[9]	
Live microsomes, Hek cell microsomes or homogenates	Hepes	Mg^{2+}	None	Sphinganine/C16-CoA	[7]	[8,13,14,20, 23,26,27]
Partially purified cellular fraction	Tris	None	Tween 20	Sphingosine/C18-CoA	[28]	[24]
HEK cell lysate	Hepes	None	Digitonin with long-chain CoA (C20 and above)	Sphinganine/C14-, C16-, C18-, C18:1-, C20-, C22-, C24- and C26-CoAs	[16]	
Yeast microsomes	Hepes	Mg^{+2}	None	Sphinganine/hexacosanoic (C26) acid and CoA[a]	[11]	
Yeast microsomes	Tris	None	Zwittergent	Sphinganine/C16-, and C26-CoA	[10]	
HeLa cell microsomes	Potassium phosphate	Mg^{2+}	None	C17-sphinganine/C16-, C18-, and C18:1-CoAs	[21]	

The table lists a number of the common published procedures for assaying CerS activity. Reactions were always performed at 37 °C, pH 7.4, except Schorling et al. [11] who assayed yeast CerS at 24 °C, pH 6.8. In some cases, a number of other factors were added to the assay reaction mixtures (such as dithiothreoitol and propylene glycol); however, the rationale behind use of these factors is not always stated, and these factors have therefore been excluded from the table.
[a]Free fatty acid and CoA were added to the reaction mixture to generate acyl CoA, in the presence of an ATP regenerating system.

Table 2
CerS proteins used in the current study, and their acyl CoA specificities

CerS	Fatty acyl CoA	References
m/hCerS1[a]	Stearoyl (C18) CoA	[13]
hCerS2	Lignoceroyl (C24) CoA	[16]
hCerS3	Lignoceroyl (C24) CoA	[19]
m/hCerS4	Stearoyl (C18) CoA and arachidoyl (C20) CoA	[14]
m/hCerS5	Palmitoyl (C16) CoA	[14]
hCerS6	Palmitoyl (C16) CoA	[16]

[a]m, mouse; h, human.

(gi45007001), 5′-CCGGATCCGCAGGGATCTTAGCCTGG, 5′-CCGAATTCTTAATCATCCATGGAGC. The PCR products and pCMV-Tag2B were digested with BamH1–EcoR1 (or EcoR1–HindIII for CerS2), ligated, and transformed into *E. coli* DH5α (Invitrogen). Vector construct inserts were verified by direct sequencing.

2.2. Cell culture and transfection

Human embryonic kidney (Hek) 293T cells and COS cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Hek cells were transfected using the calcium phosphate method, and COS cells were transfected using a jetPEI™, DNA in vitro transfection reagent (Polypus transfection).

2.3. Harvesting cells, homogenization and protein estimation

Hek cells, at ~90% confluence, were removed from culture dishes using trypsin (0.25%, w/v) followed by centrifugation (4 min, $150 \times g_{av}$). Cell pellets were then washed with phosphate buffer saline and homogenized in 20 mM HEPES–KOH, pH 7.4, 25 mM KCl, 250 mM sucrose, and 2 mM $MgCl_2$ containing a protease inhibitor cocktail (Sigma). Protein estimation was performed by the Bradford protein assay (BioRad).

2.4. Ceramide synthase assay

The assay was based on that developed for determining CerS activity in rat liver microsomes [7]. Briefly, cell homogenates were incubated with [4,5-^{3}H] sphinganine (specific activity of 15 Ci/mmol, prepared as in [7])/sphinganine (Matreya, Pleasant Gap, PA)/20 μM defatted-bovine serum albumin (Sigma), and 50 μM of the respective acyl

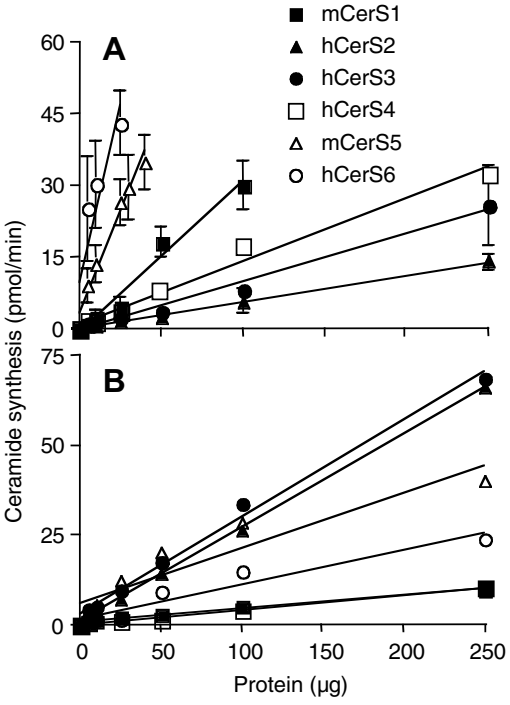

Fig. 1. CerS activity as a function of protein concentration. Homogenates were prepared from (A) Hek and (B) COS cells over-expressing CerS proteins. Assays were performed using increasing amounts of protein and 0.25 μCi of [4,5-^{3}H] sphinganine/15 μM sphinganine/20 μM defatted-bovine serum albumin, and 50 μM of the respective acyl CoA for each CerS (as shown in Table 2), for 20 min at 37 °C. Results are means ± S.D.; n = 1–5 for Hek cells, and n = 1–2 for COS cells.