Substrate specificity of rat brain ceramidase

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Abstract In this study, the substrate specificity of a newly identified rat brain ceramidase (CDase) was investigated. To this end, the major functional groups and stereochemistry of ceramide (Cer) were evaluated for their influence on the hydrolysis of substrate by this CDase. The results showed that, of the four possible stereoisomers of Cer, only the natural D-e-C_{18}-Cer isomer was used as substrate (K_m of 1.1 mol% and V_{max} of 5 μmol/min/mg). Removal of the 4–5 *trans* double bond to generate dihydroceramide decreased the affinity of the enzyme toward its substrate by around 90%, whereas changing the configuration of the double bond from the natural trans configuration into *cis* or introduction of a hydroxyl group (phytoceramide) resulted in loss of hydrolysis. Shortening the chain length of the sphingosine backbone resulted in decreased affinity. Methylation of either the primary or the secondary hydroxyl groups resulted in loss of activity. Results also indicated that Cer species that harbor long saturated or monounsaturated fatty acyl chains are preferred substrates of the enzyme. α-Hydroxylated Cer demonstrated considerably higher affinity, indicating a preference of the enzyme to those Cer molecular species. These results disclose a very high specificity of nonlysosomal CDase for its substrate, Cer.—El Bawab, S., J. Usta, P. Roddy, Z. M. Szulc, A. Bielawska, and Y. A. Hannun. **Substrate specificity of rat brain ceramidase. *J. Lipid Res.* 2002.** 43: **141–148.**

Supplementary key words nonlysosomal ceramidase • mitochondrial ceramidase • ceramide • dihydroceramide • hydroxy-ceramide • stereochemistry

Following more than a decade of investigation, it is now clear that sphingolipids play a major role in signal transduction pathways of many physiological processes including apoptosis and proliferation. In particular, the roles of ceramide (Cer), sphingosine (SPH), and sphingosine-1-phosphate (S1P) have received the highest attention (1, 2). Thus, the study of sphingolipid-metabolizing enzymes, especially those involved in the regulation of the levels of these sphingolipids, has become a major area of study.

Ceramidases (CDases) are enzymes that cleave the *N*-acyl linkage of Cer to form SPH and free fatty acid. SPH can be further phosphorylated to form S1P by the action of SPH kinase. Because of the impact that CDases can have on the regulation of the levels of these sphingolipid messengers (its products and substrate), we directed some efforts at the purification of a membrane-bound CDase from rat brain (3). It was shown that the purified enzyme was active in the neutral to alkaline range, independent of cations, and stimulated by acidic phospholipids. Based on peptide sequences of the purified enzyme, the human homolog was cloned and shown to localize to mitochondria (4).

CDase was first described by Gatt (5). In this original report, the enzyme was semipurified and characterized. Recently, several CDases have been described in tissues and cells. Acid CDase was the first to be characterized and cloned (6). Two alkaline CDases were cloned from the yeast *Saccharomyces cereviciae* (7, 8). These two enzymes appear to have distinct substrate specificity such that YDC1P selectively hydrolyzes dihydroceramide, whereas YPC1P hydrolyzes phytoceramide. A bacterial alkaline CDase was purified and cloned from *Pseudomonas aeroginosa* (9, 10), and the mammalian homolog was purified, characterized, and cloned by the same group from mouse tissues (11, 12). The mouse enzyme appears to be homologous to the human mitochondrial ceramidase (Mito-CDase), but not to the yeast enzymes. Thus, there are at least three distinct families of CDases (alkaline, acid, and nonlysosomal/mitochondrial).

Because CDases are very poorly studied and to acquire insight into the biochemistry and the physiological role of this CDase, the current study was undertaken with the aim of determining the specificity of the interaction of Cer with CDase. We focused on specific structural features required for substrate hydrolysis by this newly described enzyme. Thus, a chemical approach was followed whereby the functional groups of Cer were modified (**Fig. 1**). Cer has two asymmetrical carbons (C2 and C3) that give rise

Abbreviations: CDase, ceramidase; Cer, ceramide; Mito-CDase, mitochondrial ceramidase; NOE, N-oleoylethanolamine; OPA, o-phthaldehyde; SPH, sphingosine; S1P, sphingosine-1-phosphate; TX-100, Triton X-100.

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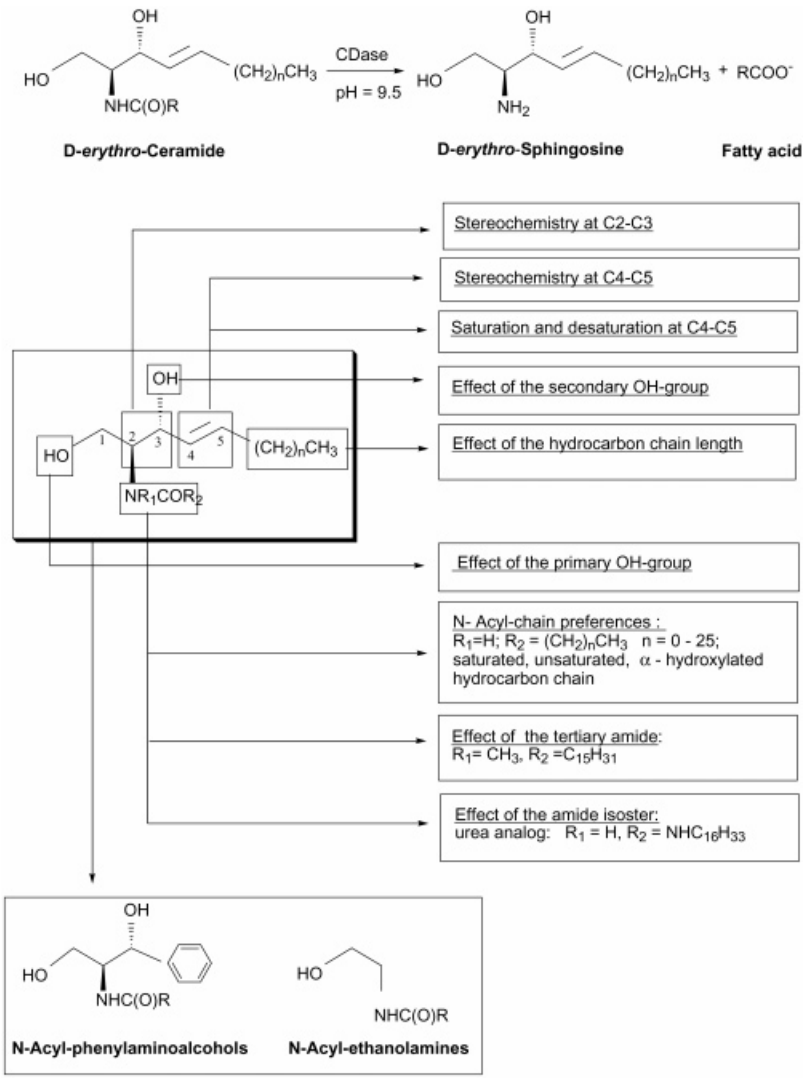

Fig. 1. Scheme of structural modifications of Cer.

to four possible isomers. In addition, Cer harbors two hydroxyl groups at positions C1 and C3. These features, in addition to other features in Cer structure, may be involved in the interaction between Cer and the enzyme. The influence of these modifications on enzyme activity was then determined. The results showed that of the four stereoisomers of Cer, only the D-erythro isomer was used as a substrate. They also indicated that the preferred substrates of the enzyme are species that harbor a long sphingoid base ($C_{18:1}$) with a *trans* double bond, and a long saturated or monounsaturated ($C_{16:0}$-$C_{18:0}$) or a very long monounsaturated ($C_{24:1}$) fatty acyl chain. On the other hand, the enzyme did not tolerate methylation of either of the hydroxyl groups at C1 or C3, or the introduction of an additional hydroxyl group in the sphingoid base. Further, methylation of the free hydrogen of the amide bond resulted in loss of hydrolysis.

In conclusion, the results indicated a strict requirement for *i*) the D-erythro isomer, *ii*) the secondary amide group, *iii*) the 4-5 *trans* double bond, and *iv*) free hydroxyl groups at the C1 and C3 positions. The implications of these results are discussed with respect to the catalytic mechanism and possible physiologic significance.

EXPERIMENTAL PROCEDURES

Materials and methods

Frozen rat brains were purchased from Pel-Freez Biologicals (Rogers, AK). Triton X-100 (TX-100) and BCA protein assay were from Pierce (Rockford, IL). *N*-oleoylethanolamine (NOE) and anandamide were from Sigma (St. Louis, MO). α-Hydroxy-Cer (Cer with hydroxy fatty acid side chain) were from Matreya (State College, PA).

All stereoisomers of Cer and modified Cer listed in **Table 1** were prepared in our laboratory as described previously (13–16).

Radiolabeled compounds, D-erythro-:[N-^{3}H]C_{16}-Cer, D-erythro-:[N-^{3}H]1-O-methyl-C_{16}-Cer, D-erythro-:[N-^{3}H]3-O-methyl-C_{16}-Cer, and D-erythro-:[N-^{3}H]N-methyl-C_{16}-Cer were prepared as described (13) starting from the corresponding sphingoid bases (15). [3-^{3}H](2*S*,3*R*)-N-[2-(1,3-dihydroxy-4*E*-octadecene)], *N*′-hexadecanene-urea [3-^{3}H]C_{16}-urea-Cer) was prepared from [3-^{3}H]SPH (14) and hexadecyl isocyanate following the procedure described for its nonradioactive analog (15).

CDase assays

CDase was isolated and purified from rat brain as described (3). Enzyme activity was determined by either one of the two following methods:

Radioactive assay. CDase activity was assayed in a mixed mi-

TABLE 1. K_m and V_{max} values of various Cer and Cer analogs

	K_m	V_{max}	V_{max}/K_m
	mol %	*μmol/min/mg*	
$C_{16:0}$-Cer	1.29	4.4	3.4
$C_{18:0}$-Cer	1.11	5.0	4.5
$C_{18:1}$-Cer	0.65	9.2	14.3
$C_{24:0}$-Cer	7.34	20.0	2.7
$C_{24:1}$-Cer	0.46	1.1	2.5
Dihydro-C_{16}-Cer	3.84	1.2	0.31
NBD-C_{12}-Cer	0.85	0.41	0.48
Keto-C_{16}-Cer	1.4	3.3	2.4
$C_{10:1}$-C_{16}-Cer	6.7	13.2	1.9
α-OH-Cer	0.13	0.239	1.85
Cis-C_{16}-Cer	NS	NS	NS
Phyto-C_{16}-Cer	NS	NS	NS
1-O-Me-C_{16}-Cer	NS	NS	NS
3-O-Me-C_{16}-Cer	NS	NS	NS
N-Me-C_{16}-Cer	NS	NS	NS

NBD, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) amino; NS, not substrate.

celle assay as described (3). Briefly, in a 200 μl reaction, [^{3}H]palmitoyl-SPH (50 μM, 0.625 mol%, ~100,000 cpm/assay) was solubilized in 100 μl glycine buffer, pH (9.5), containing TX-100. The final concentration of TX-100 in the assay was 0.5%. The reaction was started by the addition of the enzyme, and the incubation time was for 1 h. Under these conditions, the assay was linear with time and protein. The reaction was stopped by the addition of 2 ml Dole solution (isopropyl alcohol–heptane–1 N NaOH 4:1:0.1), followed by 1 ml of water and 1 ml of heptane. After centrifugation, the upper phase was discarded, and the lower phase was washed twice with heptane. Finally, 1 ml sulfuric acid (1 N) and 2 ml of heptane were added, the mixture centrifuged, and the upper phase containing the fatty acid was counted in liquid scintillation. The fatty acid was recovered in the heptane phase after removal of unreacted Cer substrate and acidification of the solution with sulfuric acid (1 N).

HPLC assay for quantitation of SPH. In some experiments, a HPLC assay was used to quantitate the released sphingoid base. The initial reaction was the same as above, but the reaction was terminated by the addition of 800 μl of chloroform–methanol (2:1 v/v) and 50 μl of sodium hydroxide (1 N). Phytosphingosine (0.5 nmol) or dihydrosphingosine (1 nmol) were added as internal standard and carrier, respectively. The mixture was then vortexed and centrifuged to allow phase separation. The lower chloroform layer was further washed with 100 μl of water, then dried and quantified for SPH using HPLC. Briefly, the o-phthaldehyde (OPA) derivative of released SPH was quantitated as described by Merrill et al. (17) and applied by us (18). The chloroform lipid residue was dissolved in methanol (50 μl), then mixed with OPA reagent (50 μl) freshly prepared [9.9 ml of boric acid (3% w/v), pH 10.5, 5 μl of mercaptoethanol, and 100 μl of ethanol containing 50 mg of OPA]. The mixture was allowed to stand for 20 min at room temperature, after which 100 μl of methanol–potassium phosphate buffer (5 mM), pH 7 (90:10 v/v) were added. An aliquote of 100 μl was injected in the HPLC. HPLC was conducted using Waters 501-HPLC model fitted with a 5 μm C18 ultrasphere ODS column (4.6 × 25) and a C18 guard column. The solvent was methanol–potassium phosphate buffer (90:10 v/v) and the flow rate was 1 ml/min. A shimadzu RF-551 spectrofluoremeter detector was used with an excitation at 340 nm and emission at 455 nm. The retention times of phytosphingosine, SPH, and dihydrosphyngosine were 12, 19, and 28 min, respectively.

One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 nmol of Cer per min.

Other procedures

Protein concentration was determined using the Bradford assay or the BCA assay for samples containing TX-100. All linear regression plots were performed using the Cricket Graph V3 program.

RESULTS

To determine the molecular determinants of interaction of CDase and its substrate Cer, we focused on key features in the Cer structure that are required for recognition by enzyme and subsequent catalysis (Fig. 1). These included the stereochemistry, the N-fatty acyl chain, the sphingoid moiety, the amide bond, and the hydroxyl groups at positions C1 and C3.

Stereochemistry

Cer contains two asymmetrical carbons giving rise to four possible stereoisomers (Fig. 1), with D-erythro (2*S*, 3*R*) serving as the naturally occurring Cer isomer. To test the stereospecificity of this enzyme, the four stereoisomers of C_{18}-Cer were synthesized and purified as reported earlier for the synthesis of C_2-Cer isomers (19).

Next, enzyme activity was assayed in a mixed micelle assay using [^{3}H]D-e-C_{16}-Cer as substrate, and competed with increasing concentrations of C_{18}-Cer isomers. The results indicated that all three isomers inhibited the hydrolysis of the substrate (used at a concentration close to the K_m value 0.63 mol%) by 50% at a concentration of 2.5 mol% (**Fig. 2A**). These results indicate that these isomers act either as substrates or inhibitors.

To distinguish these two possibilities, a HPLC assay of CDase activity was optimized by measuring the release of SPH as described (17, 18), and C_{18}-Cer isomers were used as substrates. Figure 2B shows that D-e-C_{18}-Cer was hydrolyzed by the enzyme, whereas the other isomers (L-e-C_{18}-Cer, D-t-C_{18}-Cer, and L-t-C_{18}-Cer) were inactive. The K_m for D-e-C_{18}-Cer was estimated to be 1.1 mol% and the V_{max} around 5 μmol/min/mg.

D-e-C_{18}-Cer was then used as a substrate in the assay and competed with the other isomers of C_{18}-Cer in order to determine whether the other isomers function as inhibitors. All three isomers inhibited the degradation of D-e-C_{18}-Cer with IC_{50} of 0.2–0.3 mol% (data not shown), indicating that these isomers act as inhibitors rather than as competitive substrates.

SPH moiety

The modifications shown in Fig. 1 were introduced to study the requirement of SPH structure for enzyme activity. First, the requirement for the double bond was studied. In cells, dihydroceramide, which lacks the double bond, is the precursor of Cer in the anabolic pathway of sphingolipid synthesis (20). Previously, it has been shown that dihydroceramide is biologically inactive in inhibition of cell growth compared with Cer (19). As shown in **Fig. 3A**, using the mixed micelle assay, the enzyme exhibited Michaelis–Menten kinetics when Cer or dihydroceramide were used

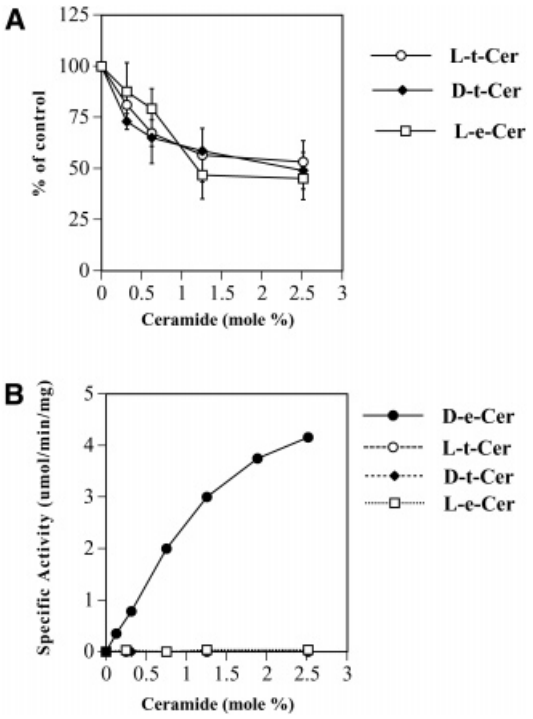

Fig. 2. Stereochemistry of Cer substrates. A: Competition of increasing concentrations of C_{18}-Cer isomers with [^{3}H]C_{16}-Cer (0.625mol%) in a mixed micelles assay (control activity was 1.8 ± 0.2 μmol/min/mg). B: CDase activity was determined using the four C_{18}-Cer stereoisomers as substrates. The HPLC assay for sphingoid products was assayed as described under Experimental Procedures. Results are average ± SD of three different experiments in A and from one experiment in B representative of two separate experiments.

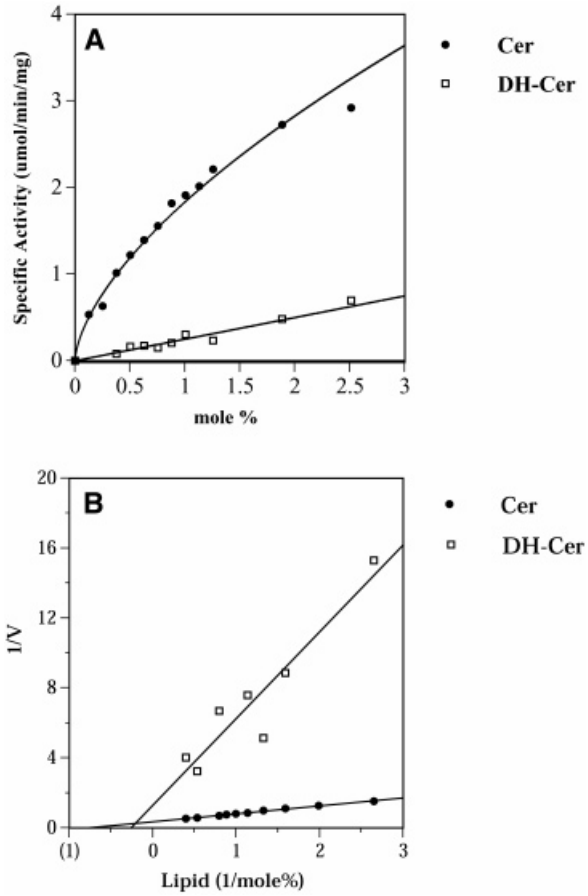

Fig. 3. Cer and dihydroceramides as substrates for CDase. A: CDase activity was measured as described under Experimental Procedures in the presence of increasing concentrations of [^{3}H]C_{16}-ceramide or [^{3}H]DH-C_{16}-ceramide. B: Lineawer-Burk plots of the data. Results are from one experiment representative of two separate experiments.

as substrates. From the double reciprocal plots (Fig. 3B), the K_m values of 1.29 mol% and 3.84 mol%, and V_{max} values of 4.4 μmol/min/mg and 1.2 μmol/min/mg were calculated for Cer and dihydroceramide, respectively. Therefore, as indicated by the V_{max}/K_m ratio (Table 1), the enzyme has 10-fold higher efficiency toward Cer than dihydroceramides, suggesting that this enzyme would preferentially cleave Cer and not dihydroceramides in vivo.

In addition, natural Cer have a trans configuration of the double bond in the sphingoid base. Thus, the D-e-C_{16}-Cer isomer having a *cis* configuration was synthesized as described (15) and used as a substrate for CDase. Using the HPLC assay and up to 200 μM of *cis*-Cer, there was no detectable SPH release (Table 1), indicating that this CDase hydrolyzes isomers having only the natural trans configuration.

Next, Cer containing SPH with a short $C_{10:1}$ chain were synthesized and assayed. Short-chain sphingoid bases ($C_{16:1}$) have been also described to be present in tissues such as liver (21). When $C_{10:1}$-C_{16}-Cer was used as substrate at various concentrations, Michaelis–Menten kinetics were observed (data not shown). The double reciprocal plot of the data was linear, and the deduced K_m and V_{max} values were 6.7 mol% and 13.2 μmol/min/mg, respectively (Table 1). These results indicate that the enzyme's affinity for the $C_{10:1}$-C_{16}-Cer was lower than that for $C_{18:1}$-C_{16}-Cer with longer sphingoid base (K_m of 1.3 mol%).

In a recent study, Mao et al. (7) reported the cloning of an alkaline CDase from yeast, termed YPC1P. Biochemical characteristics and sequence comparison of this enzyme to the human Mito-CDase showed that these two enzymes are different. Mao et al. (7) showed in their study that the yeast YPC1P preferentially hydrolyzes phytoceramides over Cer. Thus, the ability of CDase to act on phytoceramides was investigated. To this end, C_{16}-phytoceramide was synthesized and used to assay CDase activity by the HPLC method. The results indicated that phytoceramide (for up to 2.5 mol%, 200 μM) was not a substrate for the CDase (Table 1).

The SPH moiety was also investigated by replacing the sphingoid alkyl chain with an aromatic phenyl group (D-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol and L-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol; Fig. 1). To measure the activity using the phenyl-analogs of Cer, radioactive compounds labeled at the fatty acyl chain were synthesized as described (22). The enzyme did not have any activity toward these compounds, as measured by the release of the labeled fatty acid (data not shown).

Recently, fatty acid amide hydrolases have been identified to belong to the amidase family (23), and several amino acids within the active sites of amidases have also been found conserved in the fatty acid amide hydrolases enzymes (24). Sequence comparison of Mito-CDase to the human fatty acid amide hydrolase revealed that several amino acids that are critical for the activity of this enzyme are also found in the sequence of Mito-CDase, suggesting a common mechanism of hydrolysis (**Fig. 4A**). Furthermore, using the EMOTIF database (24–26), an amidase motif (GDVSPN, amino acids 337–342, not shown) was identified in the sequence of Mito-CDase. Together, these observations suggested that Mito-CDase might also belong to the amidase family or to the fatty acid amide hydrolase family. Thus, it was interesting to test whether CDase can also act on substrates of the fatty acid amide hydrolase. In fact, these substrates represent truncated Cer (amides of the fatty acyl group without the sphingoid backbone). Competition studies with anandamide and NOE were performed. Results presented in Fig. 4B show that neither fatty acyl-amide was able to compete with [^{3}H]C_{16}-Cer, suggesting that these amides are not used as substrates by the enzyme or recognized as inhibitors.

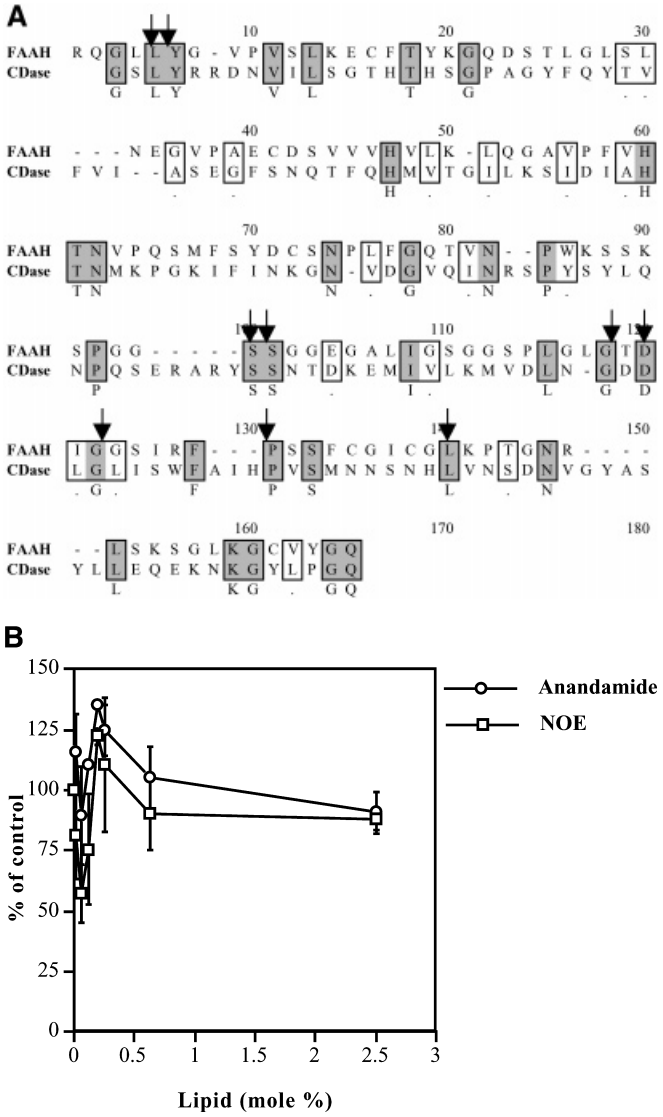

Fig. 4. Effects of fatty acyl amides on activity of CDase. A: Alignment of CDase amino acids sequences to the active site sequence of the human fatty acid amide hydrolase. Arrows indicate critical amino acids for fatty acid amide hydrolase activity. B: Competition of increasing concentrations of anandamide and NOE with [^{3}H]C_{16}-Cer (0.625mol%) in a mixed micelles assay (control activity was 1.9 ± 0.25 μmol/min/mg). Results are average ± SD of three different experiments.

On the other hand, NOE has been described to be an acid CDase inhibitor at high concentrations. Our results show that NOE at high concentration did not inhibit the CDase (Fig. 4B).

Fatty acyl group

We previously evaluated the effect of the fatty acid chain length on the enzyme activity by competing various Cers with [^{3}H]C_{16}-Cer (3). Here, we further expanded this work and studied the effect of further modifications. The HPLC assay was used to study the effect of the monounsaturation and hydroxylation of the fatty acyl chain (Fig. 1). Because *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) amino (NBD) sphingolipids have been used as substrates for various enzymes, including sphingomyelin synthase, glucosylceramide synthase, and CDase, the NBD group was also introduced at the terminal ω-amino-C12-fatty acid (16).

As shown in **Fig. 5**, when Cer containing a mixture of α-hydroxylated fatty acids (35% of α-OH-$C_{24:0}$, 24% of α-OH-$C_{18:0}$, 17% of α-OH-$C_{24:1}$) was used as substrate (up to 5 mol%), there was a release of SPH measured by HPLC, and the kinetics were hyperbolic. These results indicate that hydroxylated Cers are used as substrates by this enzyme. As Cer was a mixture of different species, an apparent K_m of 0.13 mol% and an apparent V_{max} of 0.239 μmol/min/mg could be deduced. Surprisingly, the affinity for hydroxylated Cers was 10-fold higher than the affinity for C_{16}-Cer, but the V_{max} was ~10-fold lower, giving a V_{max}/K_m ratio of 1.85, close to C_{16}-Cer (Table 1).

Next, the effect of the length and unsaturation of the fatty acyl chain was studied. C_2-Cer (0.12–2.5 mol%, 10–200 μM) did not compete with [^{3}H]C_{16}-Cer (data not shown). This result is in accordance with previous observations showing that short-chain Cers are very poor substrates, if at all. All Cers used with various saturated and monounsaturated chain lengths showed Michaelis–Menten kinetics as described in Fig. 3 for C_{16}-Cer. The Lineawer-Burk plots are represented in **Fig. 6**, and the deduced K_m and V_{max} values are presented in Table 1. The enzyme showed higher affinity for Cer containing an unsaturation in the fatty acyl chain compared with saturated Cer (the K_m values for $C_{18:1}$ and $C_{24:1}$ were 0.65 and 0.46 mol%, respectively, whereas the K_m values for $C_{18:0}$ and $C_{24:0}$ were 1.1 and 7.3 mol%, respectively). On the other hand, when NBD-C_{12}-Cer was studied, it was found to be a substrate for the enzyme with high affinity (K_m of 0.85 mol%) but with a lower rate of hydrolysis (0.41 μmol/min/mg) than observed for $C_{24:1}$-Cer, suggesting again that the introduction of unsaturation in the fatty acyl chain may increase the affinity of the enzyme for its substrate. Comparison of the V_{max}/K_m ratio of all Cer species

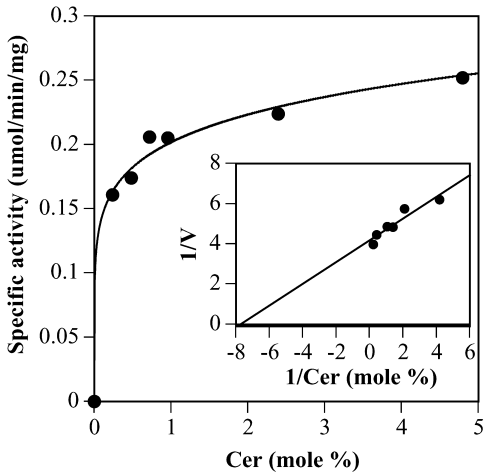

Fig. 5. Cer containing α-hydroxy fatty acids as substrates for CDase. CDase activity was carried out as described under Experimental Procedures in the presence of increasing concentrations of Cer containing a mixture of α-hydroxy fatty acids (35% of α-OH-$C_{24:0}$, 24% of α-OH-$C_{18:0}$, 17% of α-OH-$C_{24:1}$, 8% α-OH-$C_{22:0}$, 6% α-OH-$C_{23:0}$, and 1% α-OH-$C_{20:0}$). The K_m and V_{max} values represented in Table 1 were deduced using the average of molecular masses of the mixture. Results are from one experiment representative of two separate experiments.

studied (Table 1) showed that the highest activity was observed when $C_{18:1}$-Cer was used. The enzyme also preferentially used the naturally occurring Cer over NBD-C_{12}-Cer.

Amide group

To determine the importance of the secondary amide group, it was replaced with a urea group, generating the urea-Cer compound shown in Fig. 1. When urea C_{16}-Cer was used as a substrate from 0.12–2.5 mol% (10–200 μM), SPH release could not be detected as assayed by HPLC (Table 1). These results indicate a strict requirement of the amide bond for hydrolysis. The importance of the free hydrogen of the secondary amide group was also studied by synthesizing [^{3}H]N-Me-C_{16}-Cer in which the free hydrogen atom of the NHCO-group was replaced by a methyl group. The activity was measured by the release of the tritiated fatty acid, and it was found that the enzyme does not use N-Me-Cer as a substrate. These results suggest that the hydrogen atom at the NHCO-group is a critical component of the Cer structure required for catalysis.

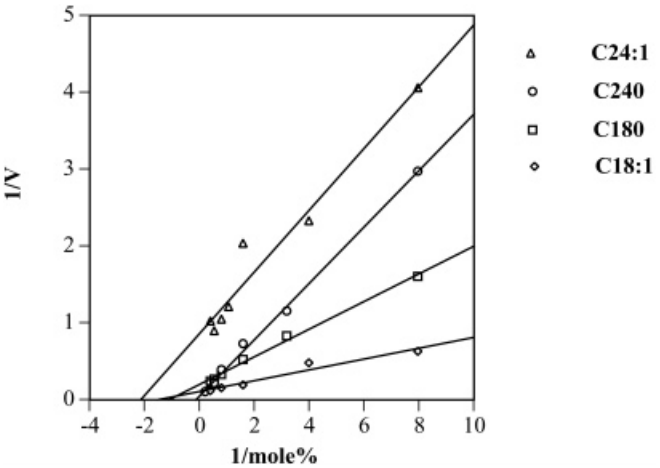

Fig. 6. Double reciprocal plots of various Cer substrates having different fatty acyl chain length. CDase activity was determined as described under Experimental Procedures in the presence of increasing concentrations of Cer substrates. The double reciprocal plots of the data are represented and the K_m and V_{max} values are shown in Table 1. Results are from one experiment representative of two separate experiments.

Primary and secondary hydroxyl groups

Previous results had shown that sphingomyelin is not a substrate for this CDase (3), indicating that bulky modification of the primary alcohol prevents hydrolysis. Here, the primary hydroxyl group was blocked by methylation, and the effect of this modification was studied on enzyme hydrolysis. 1-O-Me-Cer at concentrations up to 2 mol% was not used as substrate for CDase (Table 1). These results suggest that a free primary hydroxyl group is required for hydrolysis, and they also further support the strict specificity for Cer as a substrate.

The requirement for the secondary alcohol was also investigated. To this end, this alcohol was oxidized into ketone in [^{3}H]C_{16}-Cer, and then the generated 3-keto-[^{3}H]C_{16}-Cer was used as a substrate. The enzyme was able to hydrolyze 3-keto-[^{3}H]C_{16}-Cer following Michaelis–Menten kinetics, and the K_m and V_{max} values were similar to the values observed for $C_{16:0}$-Cer (Table 1). Thus, oxidation of this secondary group did not affect significantly the affinity or the rate of hydrolysis of Cer. However, when this secondary hydroxyl group was methylated to form 3-O-Me-C_{16}-Cer shown in Fig. 1, the enzyme failed to hydrolyze the 3-O-Me-C_{16}-Cer (Table 1) as measured by HPLC assay of the released 3-O-Me-SPH. These results indicate that the secondary alcohol is not critical for hydrolysis, but the introduction of a methyl group prevents the interaction of Cer with the enzyme and/or hydrolysis.

DISCUSSION

Understanding the interaction between Cer and CDase is important at several levels. First, it aids in understanding the biochemical mechanisms of this enzyme. Second, it can serve as a model for understanding the interaction of Cer with other enzymes or proteins, especially Cer-regulated targets. Finally, the specificity of interaction of CDase with Cer raises a number of points of possible physiologic significance. In this study, the important features required for hydrolysis of the Cer substrates by CDase were investigated through a chemical modification approach of Cer structure.

The results of this study disclose many features that are required for hydrolysis of Cer. Examination of the stereochemistry of the substrates showed that only the D-erythro isomer was hydrolyzed by the enzyme. However, all other stereoisomers were found to be inhibitors, suggesting that the stereochemistry is not crucial for the recognition of Cer by the enzyme but is crucial for the hydrolytic action.

Thus, all four stereoisomers presumably access the active site of the enzyme (to function as competitive inhibitors), but only the D-erythro isomer is in the correct spatial configuration allowing catalysis. These results begin to dissociate requirements for interaction from those of hydrolysis.

Structural modifications of the sphingoid base backbone pointed out very strict requirements for hydrolysis. Hydrogenation of the double bond reduces the affinity of the substrate (dihydroceramide) very significantly, whereas hydroxylation (phytoceramide) prevented hydrolysis. The trans configuration was also a strict requirement; *cis*-Cer was not hydrolyzed by the enzyme and did not inhibit the hydrolysis of *trans*-C_{16}-Cer. From these observations it may be concluded that the double bond is not absolutely required for catalysis, but its presence in the trans configuration allows an optimal conformation of the substrate in the catalytic site. On the other hand, the cis configuration prevents the enzyme from recognizing Cer, most probably through steric effects.

The importance of the chain length of the sphingoid base and the fatty acid was also studied. It was found that the affinity for the shorter sphingoid base, $C_{10:1}$, was lower than the most commonly occurring chain length, $C_{18:1}$. Variations in the fatty acyl chain also resulted in modification of affinity. For example, competition studies with $[^3H]C_{16}$-Cer demonstrated that the very short-chain Cer, C_2-Cer and C_6-Cer, are very poor inhibitors, whereas the inhibition with Cer having a chain length C_{10} and longer was within the same range as C_{16}-Cer. Thus, the optimal cut off for the length of the fatty acyl chain appears to be $>C_{10}$. These results are in accordance with studies on several other enzymes that act on fatty acids such as cyclooxygenases (27) and fatty acyl amide hydrolases (28), which disclose optimal chain length requirements. In the case of cyclooxygenase, structural studies disclose a pocket that optimally accommodates C_{18-20} acyl groups. Other modifications of the fatty acyl chain such as α-hydroxylation, addition of unsaturation, or addition of a NBD group increased the affinity for the substrate. Thus, the hydrocarbon chains of the sphingoid base and the fatty acid most probably interact with the enzyme through hydrophobic interactions, and impact hydrolysis by creating an optimal conformation of Cer substrate in the active site of the enzyme.

The methylation of the primary and secondary alcohol groups resulted in inhibition of hydrolysis. On the other hand, the oxidation of the secondary alcohol did not affect hydrolysis significantly, indicating that the secondary hydroxyl group does not participate as a proton donor. However, the introduction of a methyl group at this position inhibited hydrolysis, most probably by steric hindrance.

On the other hand, the free hydrogen of the amide bond appears to play a key role in catalysis, as N-Me-Cer was not used as substrate. In analogy with the serine proteases and fatty acyl amide hydrolases, the nitrogen of the amide may participate in catalysis as the proton acceptor of the sphingoid-leaving group.

At a physiologic level, the results from this study suggest a number of important attributes of this enzyme. CDase showed 10-fold higher rate of hydrolysis for Cer than for dihydroceramide, the precursor of Cer in the do novo pathway. Studies over the past decade have clearly shown that most biologic functions of Cer (such as apoptosis, growth suppression, and various signaling effects) are specific to Cer and not dihydroceramide (19). Indeed, dihydroceramides are often used as the inactive "controls" for the action of Cer. Thus, this enzyme may specifically regulate the metabolism and function of Cer and not dihydroceramide.

Furthermore, recent observations add to the significance of the specificity of Cer metabolism. For example, the recent cloning of a CDase from yeast with high specificity for dihydroceramide (dihydroCDase) (7) and a CDase with specificity to phytoceramide (phytoceramidase) (8) with mammalian homologs clearly demonstrates that individual Cer molecular species (defined by the sphingoid backbone) require specific enzymes for catabolism. Moreover, each of the CDases identified so far appears to have different intracellular localization (endoplasmic reticulum/Golgi, mitochondria, lysosomes) as well as different tissue distribution. Taken together, these studies point to compartmentalization of Cer metabolism as well as high specificity in its major species.

Further, the higher affinity of the enzyme to Cer containing α-hydroxy fatty acids may have metabolic and functional significance. α-Hydroxy fatty acid-containing Cer species are known to be particularly enriched in the complex glycolipids, and it is suggested that the α-hydroxylation is introduced after incorporation of Cer into these glycolipids (29). Although α-hydroxy fatty acid-Cer is a minor constituent in most tissues other than the nervous system, the high expression of this nonlysosomal CDase in brain raises the interesting possibility that this enzyme may preferentially function in salvage pathways, whereby it acts on α-hydroxylated Cer generated from the breakdown of complex glycosphingolipids.

In conclusion, these observations disclose a very specific interaction between Cer and CDase, suggesting that this enzyme is a specific hydrolase for Cer with long-chain sphingoid bases with the 4-5 *trans* double bond, and possibly for α-hydroxylated Cer. This specificity of the enzyme and its probable localization to mitochondria point to a unique mitochondrial pathway for the metabolism of unsaturated (and probably α-hydroxylated) Cer.

This work was supported, in part, by National Institutes of Health grant GM-43825.

Manuscript received 21 June 2001 and in revised form 10 September 2001.

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