Long-chain acyl-CoA thioesterases hydrolyze long-chain acyl-CoAs to the corresponding free fatty acid and CoASH and may therefore play important roles in regulation of lipid metabolism. We have recently cloned four members of a highly conserved acyl-CoA thioesterase multigene family expressed in cytosol (CTE-I), mitochondria (MTE-I), and peroxisomes (PTE-Ia and -Ib), all of which are regulated via the peroxisome proliferator-activated receptor α (Hunt, M. C., Nousiainen, S. E. B., Huttunen, M. K., Orii, K. E., Svensson, L. T., and Alexson, S. E. H. (1999) J. Biol. Chem. 274, 34317–34326). Sequence comparison revealed the presence of putative active-site serine motifs (GXXG) in all four acyl-CoA thioesterases. In the present study we have expressed CTE-I in Escherichia coli and characterized the recombinant protein with respect to sensitivity to various amino acid reactive compounds. The recombinant CTE-I was inhibited by phenylmethylsulfonyl fluoride and diethyl pyrocarbonate, suggesting the involvement of serine and histidine residues in the activity. Extensive sequence analysis pinpointed Ser232, Asp324, and His358 as the likely components of a catalytic triad, and site-directed mutagenesis verified the importance of these residues for the catalytic activity. A S232C mutant retained about 2% of the wild type activity and incubation with 14C-palmitoyl-CoA strongly labeled this mutant protein, in contrast to wild-type enzyme, indicating that deacylation of the acyl–enzyme intermediate becomes rate-limiting in this mutant protein. These data are discussed in relation to the structure/function of acyl-CoA thioesterases versus acyltransferases. Furthermore, kinetic characterization of recombinant CTE-I showed that this enzyme appears to be a true acyl-CoA thioesterase being highly specific for C12–C20 acyl-CoAs.

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The Peroxisome Proliferator-induced Cytosolic Type I Acyl-CoA Thioesterase (CTE-I) Is a Serine-Histidine-Aspartic Acid α/β Hydrolase*
show sequence homology only to a bile acid-CoA:aminolipid N-acyltransferase (BAAT), which catalyzes the conjugation of bile acids to glycline or taurine. Still, however, the functions of these acyl-CoA thioesterases are largely unknown.

So far, the best characterized thioesterases are thioesterases I and II, which are involved in chain termination of fatty acid synthesis by cleavage of the newly synthesized fatty acid from the fatty acid synthase complex (24, 25). These thioesterases also hydrolyze acyl-CoAs and, similar to serine proteases, lipases, and cholinesterases, have been proposed to contain a Ser-His-Asp/Glu catalytic triad, which catalyzes the ester hydrolysis. A nucleophilic serine is thought to be part of a charge-relay system along with a conserved histidine and an aspartic or glutamic acid residue (26–28). Previous site-directed mutagenesis experiments of thioesterase II have identified two of the residues that are involved in catalysis: a serine residue found within a so-called esterase consensus sequence, GXXG (29), and a histidine residue present within a GXH motif (27, 28). The GXXG motif seems to be conserved among esterases, and in mouse CTE-I Ser232 is found within a GXXGXG motif. To gain further insight into the catalytic mechanism of the type I acyl-CoA thioesterases, we have characterized recombinant CTE-I with respect to chemical modifiers and used site-directed mutagenesis to identify the catalytic amino acid residues. Our results demonstrate that CTE-I is a member of the αβ hydrolyase superfamily of esterases and that Ser232, Asp254, and His336 constitute the catalytic triad. Interestingly, the active-site serine is apparently replaced by a cysteine in the BAAT enzyme, which may mediate the different activities.

EXPERIMENTAL PROCEDURES

Identification of Putative Candidates for the Catalytic Triad of Mouse CTE-I—Fasta3 (30) (European Bioinformatics Institute server) was used for the generation of a multiple sequence alignment between mouse CTE-I and all of its homologous sequences in the GenBankTM. Each of the homologous sequences detected by Fasta3 was used independently as a query to search for homologous proteins in the three-dimensional structure protein data bank at Brookhaven. Secondary structure elements in mouse CTE-I were predicted with the PHD server (31) and predicted protein secondary structure prediction with the CCP4 (32).2

Generation of Mouse CTE-I Mutants—The full-length cDNA for mouse CTE-I was cloned into the pET16B vector (Novagen, Madison, WI) as described previously (17), producing a His-tagged fusion protein. Point mutations were introduced by PCR using the QuickChangeTM site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutagenic oligonucleotides used are shown in Table I. PCR reactions for single-base mutations were run for 30 s at 95° for at least 1 h. For the initial screening of all mutations, the effects of chemical modifiers, and sequence comparisons show that they are closely related only to BAAT, an enzyme involved in conjugation of bile acids (34, 35). Sequence analysis identified a putative active-site serine within a consensus motif (Gly-Xaa-Ser/Cys-Xaa-Gly) frequently found in the active site of αβ hydrolases (36). This superfamily of proteins is one of the largest known and includes lipases, transferases, thioesterases, haloperoxidases, lyases, etc. All of the enzymes in the αβ hydrolase superfamily share a common fold and harbor a catalytic triad formed by Ser/Cys, His, Asp/Glu. There is only one crystal structure available for a mammalian thioesterase, the palmitoyl protein thioesterase 1, shown to belong to the αβ hydrolase superfamily.
The presence of the GXXG consensus motif suggests that also the type I thioesterases may conform to the paradigm of the /H9251/H9252 hydrolase fold. Furthermore, biochemical and site-directed mutagenic analyses have provided evidence for the involvement of a serine and a histidine residue in the catalytic triad of thioesterase II, an enzyme involved in termination of fatty acid synthesis (27, 28). When mouse type I thioesterase sequences were aligned to the mouse BAAT sequence (Fig. 1), a reduced number of conserved carboxylic acid, serine, and histidine residues was identified that may constitute a catalytic triad in type I thioesterases and BAAT, although Ser232 aligned with a cysteine residue in BAAT. Fasta3 searches of the GenBank™ detected a number of proteins sharing significant overall sequence homology with mouse CTE-I (1). The alignment further suggested Ser232, Asp324, and His358 as a tentative triad, because these are the only residues of this type conserved in all of the sequences found. When each of the sequences shown in Fig. 1 was used to search for homologous proteins in the three-dimensional structure data bank, one of the Caenorhabditis elegans proteins (T16563) showed a distant but unequivocal homology with a bacterial dienelactone hydrolase of known three-dimensional structure (Protein Data Bank code 1DIN). This hydrolytic enzyme belongs to the /H9251/H9252 hydrolase superfamily, and therefore its evolutionary relationship to CTE-I confirms that this thioesterase also shares the /H9251/H9252 hydrolase fold. The sequence homology between 1DIN and CTE-I is too low to permit homology modeling, but a secondary structure prediction of CTE-I allowed us

FIG. 1. Multiple sequence alignment of mouse CTE-I and related proteins. A multiple sequence alignment was performed using Fasta3, and only proteins showing an overall sequence identity to CTE-I of more than 20% were included in the alignment. The percentage of sequence identity to mouse CTE-I is indicated at the beginning of each sequence, and the putative residues of the catalytic triad (Ser/Cys, Asp, and His) are indicated.

1, mouse CTE-I (GenBank™ accession No. O55137); 2, mouse MTE-I (Q9QYR9); 3, mouse PTE-Ia (Q9QYR7); 4, mouse PTE-Ib (Q9QYR4); 5, mouse BAAT (NP031545); 6–9, four hypothetical proteins from C. elegans (O62086, O01862, O45003, T16563) (10) putative peptidase from B. subtilis (O34493).
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Fig. 2. Secondary structure-guided partial alignment of mouse CTE-I and 1DIN. β-Strands (underlined) and α-helices (double underlined) in the structure of 1DIN (Protein Data Bank code) are indicated. Secondary structure elements predicted for CTE-I with the PHD PredictProtein program are indicated in the same manner. The asterisks denote identities, and dots indicate conservative substitutions. The nomenclature of these structurally conserved elements follows the recommendations for the α/β hydrolase superfamily (36). The residues in the triad of 1DIN are indicated by arrows.

to identify some of the central elements of the fold in CTE-I. A partial secondary structure guided alignment of 1DIN and CTE-I is shown in Fig. 2. A reduced number of conserved residues can be detected in strategic positions of the structure. Among these, Ser232, Asp324, and His358 in CTE-I aligned to the corresponding residues in the catalytic triad of 1DIN, further supporting the theory that these residues constitute the catalytic triad of CTE-I.

Effects of Chemical Modifiers on Mouse CTE-I Thioesterase Activity—The finding of a putative active-site serine was surprising, as previous characterizations of purified type I acyl-CoA thioesterases showed that the activity is not inhibited by DFP or BNPP, common serine esterase inhibitors, but is very sensitive to pCMB, a cysteine-reactive compound (18, 19). To further investigate amino acids involved in the active site, recombinant mouse CTE-I protein was produced to characterize the enzyme with respect to sensitivity to common amino acid modifying reagents. The recombinant protein was found to be insensitive to the serine reactive agents DFP and BNPP but is very sensitive to pCMB, DTT, and DTNB. The histidine-reactive reagent diethyl pyrocarbonate was also found to abolish CTE-I thioesterase activity (Fig. 3A). These results are consistent with the presence of a Ser-His-Asp/Glu catalytic triad. In addition, the cysteine reducing reagent DTT had no effect on CTE-I thioesterase activity, whereas pCMB and DTNB were found to be potent inhibitors (Fig. 3B), suggesting that a cysteine residue may be located near the active site.

Site-directed Mutagenesis and Expression of Mouse CTE-I—As discussed above, multiple sequence alignments strongly suggested that Ser232, Asp324, and His358 constitute the active-site amino acids. Therefore, PCR-based site-directed mutagenesis was performed as described under “Experimental Procedures.” The specific activity of CTE-I wild-type enzyme in the crude extract was about 140 nmol/min/mg protein, as compared with ~17 nmol/min/mg protein in non-expressing bacterial extracts (data not shown). Mutation of Ser232 to Ala, His358 to Gln, and Asp324 to Ala abolished the activity, suggesting that Ser232, Asp324, and His358 indeed constitute the active-site amino acids, whereas none of the other mutations had any major effect on activity (data not shown). Expressed His-tagged recombinant wild-type CTE-I, S232C, S232A, D324A, and H358Q were purified and characterized kinetically. Expression of the recombinant proteins were verified by SDS-PAGE (Fig. 4), and these proteins were used for further kinetic characterization. The $V_{\text{max}}$ of wild-type CTE-I was about 1.2 μmol/min/mg protein when measured with palmitoyl-CoA, with a calculated $K_m$ of about 2.6
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The primer position numbering is based on the mouse CTE-I cDNA sequence (17).

| Amino acid substitution | Primer position | Codon change | Primer sequence, 5’-3’ |
|-------------------------|-----------------|--------------|------------------------|
| S232C                   | 687–722         | TCC–TGC      | GGG CTT CTT GGG ATT TGC |
| S232A                   | 687–721         | TCC–GCC      | AAA GGG GTG GAA CTT GGG |
| D324A                   | 965–995         | GAC–GCC      | CTT AGG TCA GGA CAG CCA |
| C325A                   | 1043–1075       | TGC–GCC      | GGA GGA CCC CCA GAT CAT |
| H204A                   | 608–642         | CAC–GCC      | GGA AAC CAT GGC CAT GGA |
| H325Q                   | 965–996         | CAC–CAG      | CTT ACG TCA GGA CCA CCA |
| H373Q                   | 1113–1141       | CAC–CAA      | GGG CTT GGA GAA CGG |
| H358Q                   | 1079–1112       | CAT–CAG      | TGG TGG GTG C |
| H389Q                   | 1158–1190       | CAT–CAA      | GAG GGG CCC AAG CCT CAA |

| Amino acid substitution | Primer position | Codon change | Primer sequence, 5’-3’ |
|-------------------------|-----------------|--------------|------------------------|
| S232C                   | 687–722         | TCC–TGC      | GGG CTT CTT GGG ATT TGC |
| S232A                   | 687–721         | TCC–GCC      | AAA GGG GTG GAA CTT GGG |
| D324A                   | 965–995         | GAC–GCC      | CTT AGG TCA GGA CAG CCA |
| C325A                   | 1043–1075       | TGC–GCC      | GGA GGA CCC CCA GAT CAT |
| H204A                   | 608–642         | CAC–GCC      | GGA AAC CAT GGC CAT GGA |
| H325Q                   | 965–996         | CAC–CAG      | CTT ACG TCA GGA CCA CCA |
| H373Q                   | 1113–1141       | CAC–CAA      | GGG CTT GGA GAA CGG |
| H358Q                   | 1079–1112       | CAT–CAG      | TGG TGG GTG C |
| H389Q                   | 1158–1190       | CAT–CAA      | GAG GGG CCC AAG CCT CAA |

* The mutated codon is underlined.

Fig. 4. SDS-PAGE analysis of expressed and affinity-purified wild-type and mutant CTE-I proteins used for kinetic characterization. Wild-type and single amino acid-specific mutants of CTE-I, as indicated, were expressed in E. coli after which each protein was purified using affinity chromatography as described under “Experimental Procedures.” 2–8 μg of protein was electrophoresed in 10% polyacrylamide gels and stained with Coomassie Brilliant Blue. WT, wild type.

The specific activities of the S232A, D324A, and H358Q were about 0.1 nmol/min/mg protein (when measured with 10 μM palmitoyl-CoA), which is about four orders of magnitude lower than the activity of the wild-type CTE-I. The activities of the mutants were at the baseline detection level, and more detailed kinetic studies could therefore not be performed. Interestingly, the S232C mutant retained about 2% of the wild-type activity, suggesting that this mutant is still active as an acyl-CoA thioesterase, albeit with profoundly decreased activity. A possible explanation for the lower activity of the S232C mutant may be that the deacylation step becomes rate-limiting and that this mutant therefore becomes acylated as the acyl-enzyme intermediate. To test this possibility, we carried out experiments to compare acylation of the wild-type CTE-I and the various mutants.

Acylation of CTE-I Mutants—Acylation of CTE-I was analyzed as covalent incorporation of labeled palmitate after incubation with 14C-palmitoyl-CoA. Neither wild-type CTE-I nor the S232A mutant contained any detectable incorporation of palmitate (Fig. 5). However, the S232C mutant, which showed low palmitoyl-CoA thioesterase activity (see above) was strongly labeled with radioactive palmitate, and the H358Q and D324A mutants were also labeled, albeit much more weakly. Quantitation of the acylation (normalized to protein amount) showed that the H358Q D324A mutants contained about 14 and 10% radioactivity, respectively, of the labeling seen with the S232C mutant and the S232A mutants contained <2.6% (indistinguishable from background) of the radioactivity found in the S232C mutant. The incorporated fatty acids were covalently bound, as the labeling could be removed completely by treatment with neutral hydroxylamine (data not shown).

Acyl-CoA Substrate Specificity of CTE-I—Previous studies on CTE-I (corresponding to ACH2 of Yamada et al. (19)) showed that the enzyme is inhibited at higher substrate concentrations. We therefore tested the addition of BSA to the incubations, which at a molar ratio of 1:4.5 showed a strong protective effect against substrate inhibition (data not shown). Therefore, albumin was added at a constant molar ratio of albumin to substrate when the acyl-CoA chain length specificity was tested with acyl-CoAs longer than lauroyl-CoA (C12-CoA). When measured at 10 μM substrate, CTE-I is active on saturated acyl-CoAs of 12–20 carbon atoms, with only very low activity with decanoyl-CoA as substrate (Fig. 6). Introduction of one or two double bonds decreased the activity to about half or less compared with the corresponding saturated acyl-CoA. However, the activity is negligible with arachidonoyl-CoA (C20:4), about 35-fold lower than the activity with arachidoyl-CoA (C20:1). A summary of Vmax and Km values with various acyl-CoAs is shown in Table III. The highest Vmax values were obtained with C12–C18 saturated and monounsaturated acyl-CoAs with Km values below 4.5 μM. We also tested a number of other acyl-CoA substrates (all measured at 10 μM) of varying structures; two methyl-branched chain acyl-CoAs, 4,8-dimethylnonanoyl-CoA and 2-methylstearyl-CoA, which were both poor substrates. Also the ß-oxidation intermediates 2-trans-decenoyl-CoA and 3-hydroxypalmitoyl-CoA were much poorer substrates than the corresponding saturated acyl-CoAs. Clofibrate-CoA thioesterase activity has been reported to be localized mainly in cytosol and to be induced by clofibrate treatment in rats (39). However, CTE-I showed almost no activity with clofibrate-CoA, which excludes CTE-I as the enzyme responsible for the activity seen in rodent liver. In addition, because of the sequence similarity between CTE-I and the BAAT enzyme (which uses cholesteryl-CoA and chenodeoxycholyl-CoA as sub-
(C20:4). Activity was not detectable with acyl-CoAs of 8 or less carbon atoms in length.

Km

Experimental Procedures. Wild-type CTE-I was expressed in E. coli and purified on an affinity column as described under "Experimental Procedures." Thioesterase activity was measured with the indicated substrate at 10 μM. Stippled bars, saturated acyl-CoAs; filled bars, monounsaturated acyl-CoAs; patterned bar, linoleoyl-CoA; open bar (indicated by an arrow), arachidonoyl-CoA (C20:4).

FIG. 6. Kinetic characterization of recombinant CTE-I. Wild-type CTE-I was expressed in E. coli and purified as described under "Experimental Procedures." Acyl-CoA chain length specificity of CTE-I, measured with the indicated substrate at 10 μM. Stippled bars, saturated acyl-CoAs; filled bars, monounsaturated acyl-CoAs; patterned bar, linoleoyl-CoA; open bar (indicated by an arrow), arachidonoyl-CoA (C20:4).

Table III

Kinetic characterization of wild-type CTE-I

CTE-I was expressed and purified on an affinity column as described under "Experimental Procedures." Thioesterase activity was measured at various concentrations of straight-chain acyl-CoAs of different chain lengths (C10–C20). Activity was not detectable with acyl-CoAs of 8 or less carbon atoms in length. Km and Vmax were calculated using the Sigma Plot Enzyme Kinetics program. Recombinant CTE-I was also tested for activity with some other CoA-esters at 10 μM: 4,8-CH3-C9-CoA, 3-OH-C16-CoA, 2-CH3-C18-CoA, 2-Me-C18-CoA, 4,8-CH3-C9-CoA, 2-trans-C10-CoA, 3-OH-C16-CoA, 3-hydroxypalmitoyl-CoA, 2-CH3-C18-CoA, 2-methylstearoyl-CoA, arachidonoyl-CoA.

Acyl-CoA   \(K_m\) (μM)   \(V_{max}\) (μmol/min/mg protein)   mol/min/mg Protein
---   ---   ---   ---
C10:0   15.2   0.192
C12:0   2.6   0.780
C14:0   3.5   1.68
C16:1   1.5   1.745
C18:0   2.6   1.20
C20:0   1.1   0.621
C12:0 2-trans (cis)   2.4   0.176
C16:1 (trans)   1.8   0.188
C18:2 2-trans   4.5   0.316
C20:0   0.5   0.245
C20:4   3.0   0.007
4,8-CH3-C9-CoA   0.022
2-trans-C9-CoA   0.011
3-OH-C16-CoA   0.021
2-CH3-C18-CoA   0.014
Clofibrate-CoA   0.004
Choloyl-CoA   0

FIG. 7. Alignment of the catalytic triad amino acids of the type I acyl-CoA thioesterases and BAAT. Alignment of the GXXG motif of the active-site serine and the active-site aspartic acid and histidine residues (shown in boldface type) of the type I acyl-CoA thioesterases and the BAAT enzyme from mouse and the rat thioesterase I and II involved in fatty acid synthesis. The numbering of the mouse enzymes refers to the amino acid sequence of CTE-I. Note that the BAAT enzyme contains a cysteine residue instead of the active-site serine and that the first glycine of the GXXG motif is replaced by a serine (indicated in italics). The active-site serines of thioesterases I and II are at positions 101 and 103 respectively, and the active-site histidines are at positions 276 and 237, respectively.

In view of our observation that the activity of a recently characterized peroxisomal acyl-CoA thioesterase (PTE-2) is highly regulated by CoASH, we tested the effect of CoASH on CTE-I activity. In contrast to PTE-2, CTE-I activity was not affected at any of the concentrations tested (up to 500 μM, data not shown).

DISCUSSION

CTE-I Is a Ser-His-Asp Triad Containing α/β Hydrolase—Thioesterases are ubiquitous enzymes that appear to have diverse functions in a number of processes such as fatty acid and polyketide synthesis, removal of acyl chains from palmitylated proteins, and bioluminescence and turnover of acyl-CoA. A number of acyl-CoA thioesterases have been characterized with possible functions in lipid metabolism (for review, see Ref. 11). To date, only four thioesterases have been characterized structurally by means of x-ray crystallography. The mirestoyl acyl carrier protein thioesterase from Vibrio harveyi, which is involved in bioluminescence (40), and the mammalian palmitoyl protein thioesterase PPT1 (37) both belong to the large α/β hydrolase superfamily, which contains a catalytic Ser-His-Asp triad in the active site. However, the serine in the V. harveyi enzyme is not found in the common esterase/lipase consensus sequence Gly-X-Ser-X-Gly. Recently the structure of E. coli thioesterase II was solved (41) revealing a tertiary structure similar to β-hydroxydecanoyl thiol ester dehydratase (42) and 4-hydroxybenzoyl-CoA thioesterase (43). The catalytic site of E. coli TE II involves a novel chemistry and includes Asp204, Gln278, and Thr237, which synergistically activate a nucleophilic water molecule (41). However, the mechanism by which the 4-hydroxybenzoyl-CoA thioesterase hydrolyzes the thioester bond is not yet fully understood. In addition, thioesterases I and II of the fatty acid synthesis system have been identified as serine esterases. Site-directed mutagenesis experiments on thioesterase II have highlighted the active-site serine as well as a histidine residue, both of which are crucial for catalytic activity (26–28, 44, 45), suggesting that the active site of the thioesterases may consist of a catalytic triad similar to the Ser-His-Asp triad (276 and 237, respectively).

Alignments of the catalytic triad amino acids of the type I acyl-CoA thioesterases and BAAT.

4 M. Hunt, K. Solaas, B. F. Kase, and S. Alexson, submitted for publication.

3 P. Larsson, personal communication.

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Mutagenesis as the mutations S232A, D324A, and H358Q decreased the activity by about four orders of magnitude. The strong inhibitory effect of pCMV on wild-type CTE-I suggests the presence of a cysteine in or close to the active site. Cys\textsuperscript{378} of CTE-I is conserved in all of the type I acyl-CoA thioesterases and in the BAAT enzyme, thus being a strong candidate. However, despite several attempts we were not able to mutate this cysteine, most likely because of the difficulty in producing mutagenic primers in this area without secondary structure formation that would prevent proper PCR amplification.

Acyl-CoA Thioesterase Versus Acyltransferase Activity—CTE-I belongs to a highly conserved gene family with four more members, MTE-I, PTE-Ia, and PTE-Ib, and BAAT, the latter which is an acyltransferase involved in conjugation of bile acids. All four acyl-CoA thioesterases contain the Ser-His-Asp catalytic triad amino acids, whereas the BAAT enzyme contains a cysteine instead of the active-site serine (see Fig. 7), providing a possible mechanism for the transference activity. In addition, the first glycine of the active-site cysteine motif is replaced by a serine. Interestingly, the BAAT enzyme slowly hydrolyzes chenoyl-CoA to cholic acid and CoASH, the rate being about 2% of the BAAT enzyme, thus being a strong candidate. How- ever, despite several attempts we were not able to mutate this cysteine, most likely because of the difficulty in producing mutagenic primers in this area without secondary structure formation that would prevent proper PCR amplification.

Our finding that mutation of the active-site serine to cysteine in CTE-I almost abolished acyl-CoA thioesterase activity is similar to results obtained by others, e.g. the S203C mutant of acetyl cholineplaste (48) and the S114C mutant of the V. harveyi myristoyl-ACP thioesterase (49), which were devoid of thioesterase activity. In contrast, mutation of the active-site serine to cysteine (S101C) in the thioesterase domain of chicken fatty acid synthase and thioesterase II caused only about a 10–50% reduction in activity compared with thioesterase activities of the wild-type enzymes, with no apparent increase in acylation (26, 28, 44). It therefore seems very difficult to predict the effects on enzymatic activity of replacing the active-site serine with a cysteine residue. In most cases it appears that a cysteine residue forms a more stable acyl-enzyme intermediate, which may allow an acceptor molecule (e.g. glycine as in the case of the BAAT enzyme) to act as the second nucleophile and to complete an acyl transfer reaction. Attempts to test whether S232C CTE-I enzyme could catalyze BAAT activity failed as wild-type CTE-I is not able to hydrolyze chenoyl-CoA or chenodeoxycholoyl-CoA (data not shown), probably because of very different substrate specificities of these enzymes (see the discussion below). In an elegant study, Witkowski et al. (44) were able to engineer thioesterase II into a highly active acyltransferase by creating a S101C, H237R double mutant that was almost devoid of thioesterase activity but acted as an excellent acyltransferase (44). Thus, additional modifications may be required to engineer CTE-I into an acyltransferase. It could also be of interest to test whether CTE-I is able to conjugate fatty acids to glucose, although amidation may be more restricted to conjugation of arylacetic acids and alkyl carboxylic acids (50).

Acyl-CoA Substrate Specificity of CTE-I—Previous kinetic characterization of CTE-I (corresponding also to rat AChE of Yamada et al. (19)) have indicated that the enzyme activity drops quickly with acyl-CoAs longer than C\textsubscript{16} (19), mainly because of strong substrate inhibition. Characterization of recombinant CTE-I showed that it is also strongly inhibited at concentrations higher than -5 \mu M acyl-CoAs that have chain lengths longer than C\textsubscript{16}. However, inclusion of BSA into the assay medium largely prevented this inhibition, allowing a more extensive and detailed kinetic characterization to be carried out. CTE-I is most active on myristoyl- and palmitoyl-CoA, but also shows appreciable activity also on stearoyl- and...
arachidonyl-CoA. However, in contrast to rat ACH2, the activity of CTE-I was lower with unsaturated acyl-CoAs (irrespective of cis or trans double bonds), and in particular arachidonoyl-CoA turned out to be a very poor substrate. We also tested a number of other CoA esters, such as methyl-branched acyl-CoAs, β-oxidation intermediates, clofibril-CoA, and CoA esters of bile acids, which were all found to be very poor substrates. This is in contrast to the acyl-CoA specificity of a peroxisomal acyl-CoA thioesterase, PTE-2, which we recently characterized and showed could hydrolyze all tested CoA esters. The best substrates for PTE-2 were the bile acid intermediates chololyl- and chenodeoxycholoyl-CoA and branched chain acyl-CoA esters (51). In addition, whereas PTE-2 is inhibited by CoASH, CTE-I appears insensitive to CoASH (data not shown), indicating that CTE-I does not regulate CoASH levels but rather hydrolyzes acyl-CoAs when available. CTE-I did not show any detectable phospholipase A₉ or diglyceride lipase activities or esterase activity on nitrophenyl esters (data not shown). The narrow substrate specificity suggests that CTE-I is highly specific as a cytosolic acyl-CoA thioesterase and may also explain the apparent insensitivity to common serine esterase inhibitors, as these may not bind to the CTE-I enzyme for structural reasons, similar to results obtained with the PPT1 protein (52).

Possible Functions of CTE-I—Despite an increasing number of identified thioesterases, the functions are largely unknown. Given the importance and many functions of acyl-CoAs and fatty acids in metabolism and other cellular functions, it is obvious that acyl-CoA thioesterases may play diverse and important functions in vivo (for review, see Ref. 11). Our finding in the present study that CTE-I is not negatively regulated by CoASH suggests that CTE-I is not involved in the regulation of CoASH levels but rather that it hydrolyzes acyl-CoA when available. A cytosolic acyl-CoA thioesterase can play an important role in supply of ligands for the PPAR family of nuclear receptors (as outlined in Fig. 8). Recently the liver fatty acid-binding protein was shown to translocate to the nucleus and to interact with PPARs, and thereby liver fatty acid-binding protein may function as a cytosolic gateway for transport of fatty acids to the nucleus to be delivered to PPARs (53, 54). Two features that support such a function of CTE-I are the tissue expression and the strong and rapid regulation of expression caused by peroxisome proliferators and by fasting (22, 23). These conditions are associated with increased activation of the PPARα and increased expression of a large number of PPARα-regulated genes. Thus, the up-regulation of the CTE-I may act as an amplification system for PPARα activation. Fasting is also associated with increased flux of unesterified fatty acids into the liver and increased formation of dicarboxylic acids, the initial ω-hydroxylation being catalyzed mainly by CYP4A1, another PPARα target gene (55). The microsomal ω-hydroxylases utilizes the free fatty acid rather than the CoA ester as substrate, and therefore CTE-I may supply substrate to the ω-hydroxylases.

The kinetic characterization of the CTE-I enzyme in the present study may support yet another function for the CTE-I. The acyl-CoA substrate specificity suggests that CTE-I is mainly active on long-chain saturated acyl-CoAs, which are substrates for stearoyl-CoA desaturase 1 (SCD1). SCD1 catalyzes the introduction of a double bond, mainly in palmitic and stearic acid in the 9 position. Expression of SCD1 is highly regulated by nutritional conditions in mice, being strongly reduced by fasting⁵ and strongly increased by refeeding a fat-free diet to starved mice (56). Recent data show that disruption of the SCD1 gene largely impairs the biosynthesis of hepatic triglycerides and cholesteryl esters, which are normally rich in oleic acid (38). The dietary regulation of the CTE-I gene is largely opposite to the SCD1 gene (23), and CTE-I may therefore have a function in channeling fatty acids toward degrad-
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The Peroxisome Proliferator-induced Cytosolic Type I Acyl-CoA Thioesterase (CTE-I) Is a Serine-Histidine-Aspartic Acid $\alpha/\beta$ Hydrolase
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