Lipoamidase activity in normal and mutagenized pancreatic cholesterol esterase (bile salt-stimulated lipase)

David Y. HUI,*† Kou HAYAKAWA† and Jun OIZUMI†

*Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, OH 45267-0529, U.S.A., and †Division of Metabolism, National Children's Medical Research Center, Setagaya-ku, Tokyo 154, Japan

Purified human milk lipoamidase was digested with endoproteinase Lys-C and the digested peptides were subjected to gas-phase microsequence analysis. The sequencing of three isolated peptides of human milk lipoamidase revealed the identity of this protein with human milk bile salt-stimulated lipase (pancreatic cholesterol esterase). The identity of the cholesterol esterase with lipoamidase was confirmed by expressing a recombinant form of rat pancreatic cholesterol esterase and testing for lipoamidase activity of the recombinant protein. The results showed that the recombinant cholesterol esterase displayed both lipolytic and lipoamidase activities and was capable of hydrolysing triacetin and lipoyl-4-aminobenzoate (LPAB). The mechanisms of the esterase and amidase activities of the enzyme were further tested

by determining enzyme activity in a mutagenized cholesterol esterase with a His⁴³⁵→Gln⁴³⁵ substitution. This mutation has been shown previously to abolish enzyme activity against esterase substrates [DiPersio, Fontaine and Hui (1991) J. Biol. Chem. 266, 4033–4036]. We showed that the mutagenized protein was effective in hydrolysing the amidase substrate LPAB and displayed similar enzyme kinetics to those of the native enzyme. These data indicate that the mechanism for the cholesterol esterase hydrolysis of lipoamides is different from that of the hydrolysis of substrates with an ester linkage. The presence of an enzyme in the gastrointestinal tract capable of both ester and amide hydrolysis suggests an important role for this protein in the digestion and absorption processes.

INTRODUCTION

Pancreatic cholesterol esterase (EC 3.1.1.13), also known as bile salt-stimulated lipase, cholesteryl ester hydrolase and carboxyl ester lipase, catalyses the hydrolysis of cholesteryl esters to non-esterified cholesterol and fatty acids. The cholesterol esterase is also capable of hydrolysing other substrates such as phospholipids, lysophospholipids, retinyl esters, and mono-, di- and tri-acylglycerols [1]. The enzyme is synthesized primarily in the acinar cells of the pancreas and is secreted into the lumen of the intestine via the pancreatic duct [2]. The pancreatic secretion of cholesterol esterase is stimulated by gastric hormones in response to feeding [3]. Cholesterol esterase is also found in human milk, and it is believed to be absorbed into the digestive tract of suckling infants [4,5]. Because of its wide range of substrate specificity, cholesterol esterase has been postulated to play an important role in the intestinal absorption of dietary lipids and fat-soluble vitamins [1]. Evidence obtained from *in vitro* experiments is supportive of this hypothesis [6–10].

Another enzyme capable of catalysing the absorption of dietary vitamins is lipoamidase. This enzyme liberates lipoic acid bound to ϵ -amino groups of lysine residues in proteins such as those in the pyruvate dehydrogenase system, the α -oxoglutarate dehydrogenase system and the glycine cleavage system [11–13]. Lipoamidase displays multiple hydrolytic activities, having the ability to hydrolyse amide, ester, peptide and carbohydrate bonds [14].

A comparison of the properties of cholesterol esterase and lipoamidase has revealed many similarities between the two proteins. For example, both cholesterol esterase and lipoamidase have been demonstrated to be present in serum, liver and human breast milk [4,15–20]. Both enzymes in the liver have apparent

molecular masses of 60–70 kDa upon SDS/PAGE [19,21], while cholesterol esterase and lipoamidase activities in human breast milk are associated with proteins of apparent molecular masses of > 130 kDa [4,15,22]. Furthermore, both cholesterol esterase and lipoamidase activities are inhibited by serine-reactive reagents, indicating that serine residues are important for enzyme activity [15,23]. Analysis of the amino acid composition of cholesterol esterase and milk lipoamidase has also revealed that both proteins are enriched with proline residues [21]. These results suggest that cholesterol esterase and lipoamidase may be similar proteins. The purpose of the present investigation is to further compare the properties of these two enzymes.

MATERIALS AND METHODS

Purification and amino acid sequencing of human breast milk lipoamidase

Purification of lipoamidase was performed at 4 °C using 330 ml of human breast milk as the starting material. The purification procedure was essentially identical to that described previously for the purification of lipoamidase [15].

Purified human milk lipoamidase (single band on SDS/polyacrylamide gel) was extensively dialysed against three changes of 5 litres of distilled water, and then hydrolysed in 6 M HCl at 110 °C for 20 h in vacuum-sealed tubes. The hydrolysed product was subjected to amino acid compositional analysis by means of an h.p.l.c. amino acid analyser (Hitachi, Tokyo, Japan) with fluorimetric *o*-phthaldialdehyde detection.

The purified human milk lipoamidase was also subjected to proteolytic digestion with endoproteinase Lys-C (Boehringer Mannheim). The proteinase was incubated in pH 5.8 buffer for 15 h with the purified lipoamidase (1:50, w/w) at 37 °C. The

digested peptides were separated by reverse-phase h.p.l.c. and the eluted fragments were detected by absorbance at 220 nm. Protein sequences of the isolated peptides were determined using an Applied Biosystems 471A microsequenator.

Purification of normal and mutagenized cholesterol esterase

A 1900 bp *EcoRI*–*SacI* DNA fragment containing the entire coding region of rat pancreatic cholesterol esterase cDNA [24] was subcloned into vector pTZ18U for maintenance and propagation. The cDNA was then obtained from the recombinant plasmid by digestion with *EcoRI* and *Bam*HI, and ligated to similarly digested baculovirus transfer vector pVL1392. A mutagenized cholesterol esterase cDNA, with a glutamine-to-histidine substitution at residue 435 obtained as described previously [25], was also subcloned into the pVL1392 vector by an identical procedure. The recombinant plasmids were used to transfect *Spodoptera frugiperda* Sf9 cells [26] in the presence of *Autographa californica* nuclear polyhedrosis virus DNA. Isolation of the recombinant baculovirus, and the subsequent expression and purification of recombinant cholesterol esterase, were performed as described [27]. The normal and mutagenized forms of recombinant cholesterol esterase displayed similar electrophoretic mobilities to that of the native enzyme on SDS/polyacrylamide gels. The isolated proteins were dialysed in buffer containing 10 mM sodium phosphate, pH 6.2, 100 mM NaCl, 0.02 % sodium azide, 1 mM EDTA and 1.5 % glycerol. The purified proteins were stored at -70°C until use.

Enzyme activity assays

Activity was assayed in buffer containing 100 mM sodium phosphate, pH 7.0, 1 mM EDTA and 10 mM 2-mercaptoethanol at 37°C [14]. Usually, 45 μl of substrate-containing buffer was mixed with 5 μl of enzyme solution. The reaction was stopped by addition of 50 μl of 0.1 M HCl. An aliquot of 10 μl was injected directly into the reverse-phase h.p.l.c. system [14]. Lipoamidase (lipoyl-X hydrolase) activity was measured by using lipoyl-4-aminobenzoate (LPAB) as the substrate. The reaction product, 4-aminobenzoate, was detected by intrinsic fluorescence [16]. Cholesterol esterase and lipolytic activities were characterized by the hydrolysis of triacetin; the reaction was monitored by the liberation of acetic acid. The product was measured by the salting-out reverse-phase h.p.l.c. method as previously described [14], except that acetate was detected by u.v. absorption at 210 nm. Esterase activity was measured by the conversion of *p*-nitrophenyl acetate into *p*-nitrophenol [28]. Kinetic parameters were calculated as described previously [17]. In order to calculate k_{cat}/K_m values, the molecular mass of cholesterol esterase/lipoamidase was assumed to be 76 kDa.

RESULTS

Lipoamidase purified from human milk as described previously [17] was subjected to amino acid analysis and the results were compared with the nucleotide sequence-deduced amino acid compositions of human milk bile salt-stimulated lipase [5] and rat pancreatic cholesterol esterase [24] (Table 1). The results indicated that the amino acid compositions of these proteins are very similar. Human milk lipoamidase was then subjected to Lys-C peptidase hydrolysis and the digested peptides were separated on an h.p.l.c. column. Three individual polypeptides

were isolated for sequence analysis. As shown in Table 2, the amino acid sequences of all three peptides from human milk lipoamidase were identical to sequences within the human cholesterol esterase (bile salt-stimulated lipase). Because both lipoamidase and cholesterol esterase are hydrolases with multiple substrate specificities, it is possible that the lipoamidase and cholesterol esterase activities in human milk reside in the same protein.

The possible identity between human milk lipoamidase and cholesterol esterase was investigated by determining lipase activity in the purified lipoamidase preparation. We have chosen to use triacetin, instead of cholesteryl esters, as the substrate for measuring lipase activity in this experiment. The cholesterol esterase-catalysed hydrolysis of the former substrate does not have an absolute requirement for the presence of bile salts, thereby affording direct comparison of the lipolytic activity with the amidase activity of the enzyme. The results (Figure 1)

Table 1 Amino acid compositions of lipoamidase and cholesterol esterases

The amino acid composition of purified human lipoamidase (milk LIP) was determined [17]. The amino acid compositions of human milk bile salt-stimulated lipase/cholesterol esterase (human BSSL/CEH) and rat cholesterol esterase (rat CEH) were derived from the nucleotide sequence data.

	Composition (mol %)		
	Milk LIP	Human BSSL/CEH	Rat CEH
Asx	11	11	11
Thr	8	9	8
Ser	5	6	5
Glx	7	5	9
Pro	17	14	8
Gly	9	10	9
Ala	9	10	10
Val	8	8	7
Met	0.5	1	2
Ile	4	4	6
Leu	7	5	7
Tyr	3	3	4
Phe	4	2	4
Lys	5	5	5
His	1	1	2
Arg	2	3	3

Table 2 Sequence comparison of human milk lipoamidase peptides with cholesterol esterase

Human milk lipoamidase was purified and subjected to proteolytic digestion with endoproteinase Lys-C as described in the Materials and methods section. The digested peptides were separated on an h.p.l.c. column and then sequenced with a microsequenator. The sequences were compared with the amino acid sequence of human milk cholesterol esterase (CEH) as reported in [5]. The (Lys) residue preceding each lipoamidase peptide sequence was predicted based on Lys-C digestion of the protein.

Peptide	Sequence
Lipoamidase peptide 1	(Lys) – Ile Pro Phe Ala Ala Pro Thr
CEH residues 31–39	Lys Gly Ile Pro Phe Ala Ala Pro Thr
Lipoamidase peptide 2	(Lys) Ala Leu Glu Asn Pro Gln Pro His Pro Gly Trp Gln Thr Leu Lys
CEH residues 40–56	Lys Ala Leu Glu Asn Pro Gln Pro His Pro Gly Trp Gln Thr Leu Lys
Lipoamidase peptide 3	(Lys) Leu Val Ser Glu Phe Thr Ile Thr
CEH residues 346–354	Lys Leu Val Ser Glu Phe Thr Ile Thr

Figure 1 Hydrolysis of LPAB (○) and triacetin (△) by human milk lipoamidase

Purified human milk lipoamidase (1 mg/ml) was incubated with substrate buffer as described in the Materials and methods section. Substrate concentrations were 0.25 mM LPAB and 10 mM triacetin. The results shown here were obtained by averaging duplicate assays from a single experiment. The variations in enzyme kinetic data from several experiments using different enzyme preparations were similar to those summarized in Table 4.

Table 3 Effect of bile salt on lipoamidase hydrolysis of *p*-nitrophenyl acetate

Purified human milk lipoamidase (32 μg) was incubated with *p*-nitrophenyl acetate in the presence or absence of taurocholate for 2 h at 37 °C. At the end of the incubation period, the amount of product (*p*-nitrophenol) released from the substrate was quantified by measuring the absorbance at 405 nm as described [28].

Taurocholate (mM)	Specific activity (nmol/min per mg of protein)	Activity (% of control)
0	27.4	100
2	44.8	164
10	66.0	241

confirmed the lipase activity of the purified lipoamidase and showed the efficient hydrolysis of triacetin as well as of LPAB. In fact, the enzyme was 8-fold more efficient in the hydrolysis of the

Figure 2 Hydrolytic activities of recombinant rat cholesterol esterase and mutant rat cholesterol esterase

Purified recombinant enzymes (1 mg/ml) were used and assayed as in Figure 1. Other conditions were described in the Materials and methods section. Open and closed symbols represent recombinant rat cholesterol esterase and mutant recombinant rat cholesterol esterase respectively. Circles and triangles represent hydrolysis of LPAB and triacetin respectively. The results shown were obtained from duplicate assays from one experiment. The variations in enzyme kinetic data from several experiments using different enzyme preparations were similar to those summarized in Table 2.

lipase substrate triacetin ($V_{\max}$, 23.3×10^5 pmol/min per mg) than of the amidase substrate LPAB ($V_{\max}$, 3.2×10^5 pmol/min per mg). Purified milk lipoamidase was also capable of hydrolysing the water-soluble artificial substrate *p*-nitrophenyl acetate. The latter activity was increased several-fold when the assay was performed in the presence of taurocholate (Table 3). The bile salt activation of *p*-nitrophenyl acetate hydrolysis was similar to that observed previously for human pancreatic cholesterol esterase [28], further suggesting identity between the milk lipoamidase and the cholesterol esterase.

Consideration was given to the possibility that the above results were due to contamination of the lipoamidase preparation with cholesterol esterase, an abundant protein in human breast milk. Therefore we took advantage of the availability of an efficient expression system for recombinant rat cholesterol esterase and determined whether the recombinant enzyme exhibits both cholesterol esterase and lipoamidase activities. Although the recombinant rat cholesterol esterase was less active with

Table 4 Enzyme kinetics of recombinant normal and mutagenized cholesterol esterases

Recombinant rat cholesterol esterase and the His⁴³⁵ → Gln⁴³⁵ mutant were expressed and purified from baculovirus-infected Sf9 cells as described in the Materials and methods section. The abilities of the recombinant proteins to hydrolyse the lipoamidase substrate LPAB and the lipase/esterase substrate triacetin were compared. The values reported here represented the averages $\pm$ S.D. of duplicate determinations from three separate experiments. The K_m and $V_{\max}$ for LPAB hydrolysis are reported in units of μ M and pmol/min per mg respectively; the K_m and $V_{\max}$ for triacetin hydrolysis are reported in units of mM and nmol/min per mg respectively. N.R., no reaction.

Substrate	Native cholesterol esterase			Mutant cholesterol esterase		
	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	K_m	$V_{\max}$	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	K_m	$V_{\max}$
LPAB	2.12 ± 0.03	24 ± 2.2	45.5 ± 4.3	1.88 ± 0.15	11 ± 1.2	18.5 ± 1.7
Triacetin	28.0 ± 3.4	15 ± 0.9	323 ± 26	N.R.	N.R.	N.R.

regard to both lipase and amidase activities than the purified human milk enzyme, it was capable of hydrolysing both triacetin and LPAB (Figure 2). The lower hydrolytic activity of the recombinant rat cholesterol esterase compared with the human milk enzyme is consistent with previously published data showing higher activity of human cholesterol esterase than of rat pancreatic cholesterol esterase [29].

To determine if the enzymic hydrolysis of ester and amide substrates occurs through a similar mechanism, we took advantage of our previous observation that cholesterol esterase with a His⁴³⁵→Gln⁴³⁵ substitution was inactive with regard to esterase activity and could not hydrolyse either *p*-nitrophenyl butyrate or cholesteryl oleate, even in the presence of bile salts [25]. In the present study we showed that the mutant cholesterol esterase was also ineffective in the hydrolysis of the lipase substrate triacetin (Figure 2). However, when the mutant protein was used to determine amidase activity, significant hydrolysis of LPAB was observed (Figure 2). The kinetics of LPAB hydrolysis by the native and mutant cholesterol esterases are compared in Table 4. The His⁴³⁵→Gln⁴³⁵ substitution in cholesterol esterase was found to have a minimal effect on the K_m of the reaction, but reduced the V_{max} , approx. 2.5-fold.

DISCUSSION

Lipoic acid is a coenzyme of the pyruvate dehydrogenase system and the α -oxoglutarate dehydrogenase system [11]. It also serves as a coenzyme in the enzyme system that cleaves glycine into carbon dioxide and a methyl group [30,31]. The lipoic acid is usually found to be covalently bound to protein moieties through lysine residues. The liberation of lipoic acid from this complex requires the enzyme lipoamidase. Lipoamidase activity has been found in a variety of mammalian tissues, including human breast milk [15], human serum [16], pig brain [17] and guinea pig liver [21].

In an effort to further characterize lipoamidase(s), experiments were initiated to determine the structure of the human milk protein. The results presented in this and previous papers demonstrate that human milk lipoamidase is similar to human cholesterol esterase (bile salt-stimulated lipase) in many respects. For example, human milk lipoamidase has an identical molecular mass to that of cholesterol esterase in human milk. The amino acid sequences of three proteolytic peptide fragments of lipoamidase are also identical to sequences that occur in human milk cholesterol esterase. More importantly, lipoamidase purified from human milk displayed high lipolytic and esterase activities (Figure 1, Tables 1–3), similar to those observed for human pancreatic cholesterol esterase [32]. Finally, recombinant cholesterol esterase expressed in baculovirus-infected insect cells displayed significant lipoamidase activity. Based on these observations, we conclude that the lipoamidase and cholesterol esterase hydrolytic activities in human milk are catalysed by the same protein.

An interesting observation was made by comparing the lipoamidase and esterase activities in a mutant protein containing a His⁴³⁵→Gln⁴³⁵ substitution. The results showed that esterase activity is dependent on the presence of histidine at residue 435. This histidine residue was postulated to form part of the catalytic triad of cholesterol esterase [25]. However, experiments presented in the present paper reveal that substitution of glutamine for histidine at residue 435 resulted in an enzyme that remained capable of hydrolysing lipoamidase substrates, albeit with reduced K_m and V_{max} . The k_{cat}/K_m values for hydrolysis of LPAB were similar in the native and mutagenized proteins. These

results are consistent with previous observations that modification of lipoamidase with histidine-specific reagents, such as diethyl pyrocarbonate, resulted in only a 20% inhibition of activity [17]. In contrast, modification of cholesterol esterase with similar reagents abolished the lipolytic and esterase activities of the protein [33]. Taken together, these data suggest that the mechanism by which cholesterol esterase catalyses lipoamide hydrolysis is different from that causing the hydrolysis of substrates with an ester linkage. However, it is important to note that both cholesterol esterase and lipoamidase activities in the protein require a functional serine residue. Chemical modification of the protein with di-isopropylfluorophosphate abolished both lipoamidase [15] and cholesterol esterase [23,33] activities. The precise mechanism by which this protein catalyses lipoamide hydrolysis remains to be determined.

Although the data presented in this paper indicate that human milk lipoamidase is identical to cholesterol esterase (bile salt-stimulated lipase), it is unlikely that this is the only protein with lipoamidase activity. As stated above, previous studies have demonstrated that lipoamidase may be a ubiquitous protein with a wide tissue distribution. However, the bile salt-dependent cholesterol esterase has been found only in pancreas [1], liver [18,19], serum [18] and human milk [4]. Cholesterol esterase mRNA was not present in the brain (D. Y. Hui, unpublished work), a tissue with abundant lipoamidase activity [17]. Furthermore, lipoamidases purified from pig brain and guinea pig liver have substantially different amino acid compositions than that of the cholesterol esterase of either rat or human origin [17]. Therefore it is quite possible that lipoamidase activity in other tissues is due to other proteins with similar activities. In this respect, non-specific esterases are ubiquitously present in all mammalian tissues. These non-specific esterases have structural domains that are very similar to those found in cholesterol esterase [34]. It would be interesting to determine whether lipoamidase activity in other tissues is derived from these non-specific esterases.

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