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Bile salt activation of human cholesterol esterase does not require protein dimerisation

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Abstract Human milk cholesterol esterase (bile salt-activated lipase) plays a role in the dietary uptake of triacylglyceride and cholesteryl ester. The activities toward these substrates are mediated through a unique bile salt-activated mechanism. Previously, it has been proposed that a necessary step in this process is prior protein dimerisation in the presence of primary bile salts. In this study, we addressed the role of protein dimerisation by investigating bile salt interactions on full length and truncated recombinant forms, as analysed by size exclusion chromatography and concanavalin A Sepharose binding experiments. The present findings demonstrate that protein dimerisation is not an obligatory component of the bile salt-activated pathway. A new functional role for the glycosylated C-terminal domain in cholesterol esterase is also demonstrated in the prevention of non-specific hydrophobic interactions.

Key words: Cholesterol esterase; Lysophospholipase; Carboxylester lipase; Bile salt activation; Bile salt-stimulated lipase

1. Introduction

Cholesterol esterase (bile salt-activated lipase) belongs to the cholinesterase family of proteins and hydrolyses a broad range of substrates including triacylglycerides and cholesteryl esters [1]. It is secreted by the exocrine pancreas and by a number of species in the milk where it ensures efficient lipid uptake in the newborn. As shown from cDNA cloning, the milk and pancreatic forms of cholesterol esterase are derived from the same gene [2]. Cholesterol esterase has also been identified in plasma [3] and evidence suggests it is involved in lipoprotein metabolism [4,5]. Recently, its role in the uptake of dietary cholesteryl ester has been established by studies with gene knockout mice [6].

Cholesterol esterase possesses a unique bile salt activation mechanism in which the hydrolysis of emulsified long chain triacylglyceride and cholesteryl esters is dependent on primary bile salts [1]. This phenomenon has been explained in terms of a protein activation site that recognises specifically the 7α-hydroxyl group of primary bile salts [7]. However, this group is not the sole structural component required for activation, as demonstrated with CHAPS. This bile salt analogue binds to cholesterol esterase without causing activation, presumably by preventing subsequent interfacial binding to a lipid emulsion [8]. Therefore, other steroidal side chains are also important for enzyme/substrate interactions [8].

Based on early evidence, it has been proposed in terms of a two site model that bile salt activation proceeds through protein dimerisation [7,9]. Here, binding of primary bile salts at the activation site induces dimerisation of cholesterol esterase and subsequent activation [7]. In contrast, secondary bile salts, which do not activate and lack a 7α-hydroxyl group, bind at a distinct lipid-binding promoting site without causing dimerisation [7]. Recently, a molecular interpretation for protein dimerisation has been advocated, based on a computer-generated tertiary structure for the salmon isoform (carboxylester lipase) modeled on the related acetylcholinesterase tertiary structure [10].

Despite a two site interpretation of bile salt binding and evidence for bile salt-induced conformational changes [11], whether protein dimerisation is integral to the activation mechanism has not been conclusively established. In the present study, the role of protein dimerisation was addressed by investigating bile salt interactions on full length and truncated recombinant forms of cholesterol esterase. The fully active truncated mutant lacked a novel glycosylated C-terminal domain [12], comprising a proline-rich repetitive consensus sequence [2]. By studying the effects of bile salts on these recombinant forms using size exclusion chromatography and concanavalin A Sepharose binding, the present findings demonstrate that protein dimerisation is not required for bile salt activation. Furthermore, differences observed between the two recombinant forms implicate a new functional role for the glycosylated C-terminal domain in counteracting non-specific hydrophobic interactions.

2. Materials and methods

2.1. Cloning and expression

cDNA sequences encoding full length (residues 1–722) and truncated cholesterol esterase (residues 1–518) were cloned from a λgt10 cDNA library constructed from lactationally competent human breast tissue (Clontech), as described previously [12]. The truncated variant lacked the glycosylated C-terminal domain starting from position 536 and a preceding polypeptide, Leu519–Gln535. Due to differential glycosylation [13], both the truncated and full length recombinant forms are secreted from their respective stable cell lines as various protein bands, as analysed by SDS-PAGE. These are 58 kDa and 63 kDa for the truncated recombinant form and 115 kDa/117 kDa and 120 kDa for the full length recombinant form.

2.2. Purification of recombinant cholesterol esterases

Recombinant cholesterol esterases were purified as described previously [12] with one modification. Namely, after anion exchange (Q-Sepharose, Pharmacia), active fractions were loaded onto a heparin Sepharose step (Hep-Pac cartridge, Pharmacia) in 10 mM sodium phosphate, pH 7.6, and the recombinant cholesterol esterases eluted with a NaCl gradient. No differences between the elution positions between the full length and truncated recombinant forms were observed. The purified proteins were concentrated by ultracentrifugation (Amicon) and analysed by SDS-PAGE.

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2.3. Concanavalin A binding

Either full length or truncated recombinant forms were loaded onto concanavalin A Sepharose (Pharmacia) equilibrated with 10 mM sodium phosphate, pH 7.6, with various concentrations of NaCl or CHAPS as indicated in the figure legend. Of the recombinant forms, the 63 kDa (truncated) and 120 kDa (full length) species respectively were retained by the column, compatible with the presence of protein *N*-linked glycosylation. After complete elution of unbound material, these *N*-glycosylated species were eluted with 0.2 M methyl-α-D-glucopyranoside. For the binding experiments, eluted proteins were concentrated by ultracentrifugation (amicon) and analysed by SDS/PAGE using 10% or 7.5% acrylamide gels for truncated and full length recombinant forms respectively.

3. Results and discussion

3.1. Bile salt interactions with human cholesterol esterase

To investigate bile salt interactions, size exclusion chromatography was performed on the truncated mutant in the presence of the primary bile salt sodium cholate (Fig. 1). Interestingly, at a submicellar concentration of sodium cholate, sufficient to activate enzyme activity (2 mM), there was no change in the apparent molecular mass exhibited by the truncated mutant at approximately 61 kDa (major peak). Nor was there any change when sodium cholate was substituted for identical concentrations of the secondary bile salt, sodium deoxytaurocholate, or the bile salt analogue CHAPS. The minor peak at approximately 80 kDa is attributable to traces of transferrin originally present in the cell culture media, as indicated by SDS-PAGE. Identical results were also obtained with the full length recombinant cholesterol esterase. Thus, submicellar concentrations of either these bile salts, or the bile salt analogue, CHAPS, had no effect on the apparent molecular mass at approximately 300 kDa (not shown).

These results resolve conflicting ultracentrifugation evidence concerning the effect of submicellar primary bile salts on the molecular size of the enzyme [7,14]. Rather, as both primary and secondary bile salts cause cholesterol esterase to bind to emulsion particles [8], bile salt activation is likely mediated through conformational factors. This alternative mechanism is supported by circular dichroism spectropolarimetry on the rat isoform [11].

The interaction of cholesterol esterase with micellar bile salt has also received attention in the literature [9,10]. On the basis of chemical modification protection studies, it has been proposed that a functional micellar binding site is formed from a premicellar bile salt binding site through dimerisation. As micellar bile salt could potentially interact differently than the dispersed bile salt form, this possibility was investigated.

For the full length recombinant form, micellar concentrations (10 mM) of either sodium cholate, sodium deoxytaurocholate, or the bile salt analogue CHAPS, shifted the apparent molecular mass from 300 kDa to approximately 510 kDa (not shown). However, the possibility that this molecular mass shift was due to protein dimerisation was discounted on the basis of identical experiments with the truncated mutant in which the apparent molecular mass increased from 61 kDa to approximately 70 kDa (Fig. 1). Reinjection of the 70 kDa peak onto the column re-equilibrated without bile salt partially resolved both 70 kDa and 61 kDa peaks suggesting an interaction between the protein and the bile salt micelle (not shown). These results strongly suggest that cholesterol esterase does not dimerise in the presence of micellar bile salt. Instead, the observed molecular mass shift for the full length enzyme is likely due to the non-globular behaviour of the solvated protein [15] conferred by the C-terminal glycosylated domain.

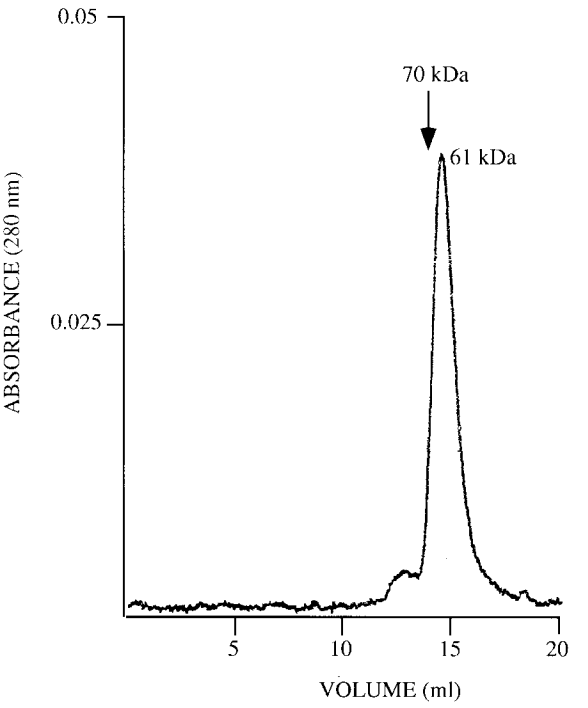

Fig. 1. Bile salt interactions with the truncated mutant. Truncated recombinant cholesterol esterase (60 μg protein) was resolved on a Superose-12 column (Pharmacia) equilibrated with 10 mM sodium phosphate, pH 7.6, +0.1 M NaCl. In the absence or presence of submicellar (2 mM) sodium cholate, the truncated mutant exhibited an apparent molecular mass at 61 kDa (major peak). Micellar sodium cholate (10 mM) shifted this peak position to an apparent molecular mass of 70 kDa, as indicated by the arrow (trace not shown).

Additional support that protein dimerisation does not occur was provided by concanavalin A binding studies of differentially glycosylated full length forms (Fig. 2, right panel). As indicated by SDS-PAGE, the full length recombinant cholesterol esterase is secreted from the mammalian cell culture system as three protein forms: a 120 kDa species which is immobilised by concanavalin A sepharose; and two 115/117 kDa concanavalin A non-binding forms. If dimerisation occurs in the presence of primary bile salts, it would be possible to detect an association between these various glycosylated forms. However, no association was observed under conditions in which bile salt activation occurs (far right). Thus, a functional micellar binding site is not formed through dimerisation as originally proposed [9].

3.2. Role of the C-terminal glycosylated domain in the prevention of aggregation

An enigma particular to cholesterol esterase is how protein aggregation in the inactive state is prevented. For many other characterised lipases displacement of a 'lid' overhanging the active site can augment exposure of hydrophobic surfaces surrounding the active site cavity [16,17]. In the closed form, aggregation is largely prevented through shielding of hydrophobic patches from the bulk solvent [18]. For cholesterol esterase, however, structural predictions [10] and limited proteolysis [12] suggest the presence of permanently exposed

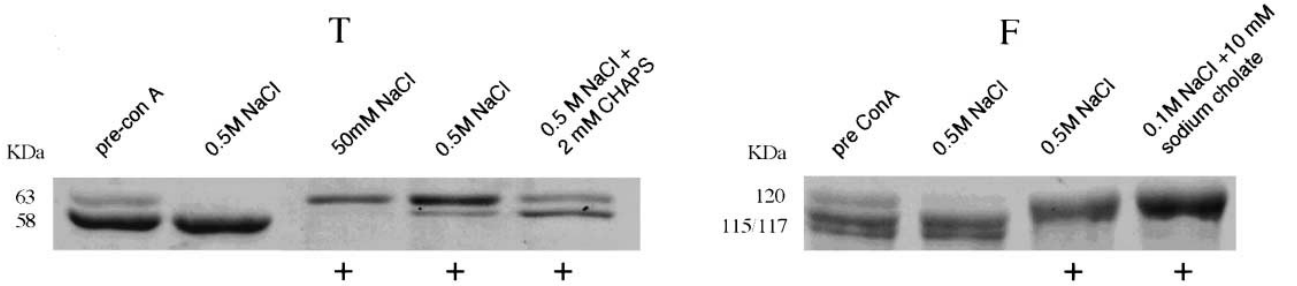

Fig. 2. Concanavalin A Sepharose binding of full length (F) and truncated (T) recombinant forms. Left panel: Association of 58 kDa and 63 kDa truncated forms (5 μg total protein in each lane). From left: 58 kDa and 63 kDa truncated forms prior to loading onto concavalin A; unbound 58 kDa form; elution of the 63 kDa form with 0.2 M methyl-α-D-glucopyranoside (indicated by + signs) in the presence of 50 mM NaCl, 0.5 M NaCl, and 0.5 M NaCl+2 mM CHAPS. Right panel: Separation of full length glycosylated recombinant forms. From left: glycosylated forms prior to loading onto concanavalin A; unbound 115/117 kDa forms; elution of 120 kDa form with 0.2 M methyl-α-D-glucopyranoside (indicated by + signs) in the presence of 0.5 M NaCl, or 0.1 M NaCl+10 mM sodium cholate.

hydrophobic surfaces, raising the possibility of protein aggregation via hydrophobic interactions.

Compatible with the presence of exposed hydrophobic surfaces, concanavalin A sepharose binding experiments and size exclusion chromatography suggested that cholesterol esterase undergoes partial aggregation in the absence of the glycosylated C-terminal domain. In the cell culture expression system the fully active truncated mutant is expressed as 58 kDa and 63 kDa protein forms, as measured by SDS/PAGE (Fig. 2, left panel) [12]. The 63 kDa species arises from carbohydrate at an *N*-linked site (Asn-187) as shown by the generation of the 58 kDa form by *N*-glycosidase F cleavage [13]. Purification by concanavalin A sepharose effectively separates these two forms with specific elution of the bound 63 kDa N-linked glycosylated species by 0.2 M methyl-α-D-glucopyranoside (left panel).

In the presence of 0.5 M NaCl, a small fraction of the 58 kDa form was associated with the 63 kDa species (left panel). The possibility was considered that this association was an artifact, due either to alternative glycosylation, or a non-specific interaction of the 58 kDa form with the column matrix. However, an artifact was incompatible with the absence of non-specific binding under similar conditions in size exclusion chromatography, and with the loss of the 58 kDa form at a lower NaCl concentration (50 mM) (left panel). Interestingly, the association between the 58 kDa and 63 kDa recombinant forms was enhanced in the presence of 2 mM CHAPS. A possibility is that CHAPS binds to cholesterol esterase and enhances aggregation via an increase in hydrophobic surface area.

Further supportive evidence for salt-dependent partial aggregation was obtained by size exclusion chromatography (Fig. 3). Under conditions otherwise identical to Fig. 1, two additional higher molecular mass species appeared reproducibly in the presence of 0.5 M NaCl, suggesting the existence of aggregated states. One of these peaks appeared near the void column volume while the other corresponded to an apparent molecular mass of approximately 126 kDa.

Under these conditions, an aggregated state could be energetically favourable, in which normally exposed hydrophobic surfaces are shielded from the aqueous solvent. Interestingly, a similar phenomenon is observed with the distantly related fungal lipase from *Candida cylindracea* [19] which exists in equilibrium with dimeric and monomeric forms [20]. Recent structural analysis has revealed a dimeric association of monomers in which the two active-site cavities face each other, shielding hydrophobic surfaces from the aqueous environment [21].

The partial aggregation of the truncated recombinant mutant indicates a further role for the C-terminal glycosylated domain. This region has attracted much interest from several laboratories [12,13,22,23]. Although catalytically non-essential, they appear to be involved in soluble heparin interactions [23] and mediate substrate delivery [22]. Despite one report [24], they protect the protein molecule against proteolytic degradation by pancreatic proteases [12]. In the present study, partial aggregation was not exhibited by the full length recombinant form. This comparative difference indicates that the glycosylated C-terminal domain counteracts a tendency for cholesterol esterase to self-aggregate.

In conclusion, the present results refocus an aspect of bile

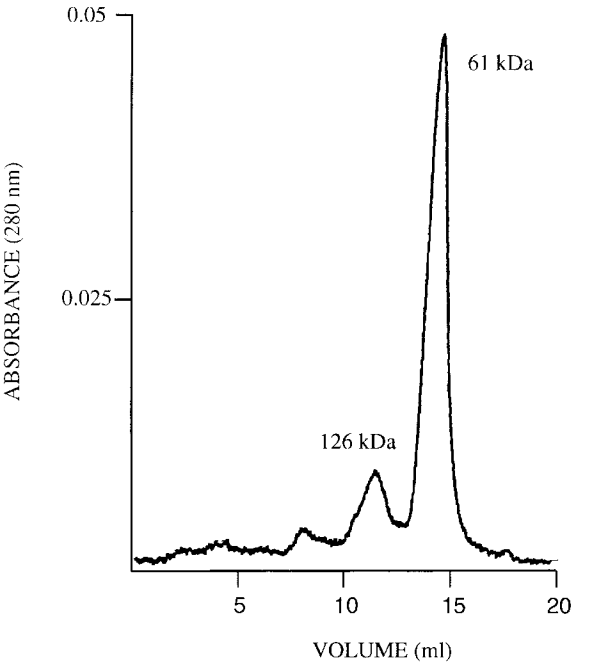

Fig. 3. Aggregrated forms of the truncated recombinant mutant. The truncated recombinant mutant (60 μg protein) was resolved on a Superose-12 column (Pharmacia) equilibrated with 10 mM sodium phosphate, pH 7.6, +0.5 M NaCl. Under these conditions, two higher molecular mass species appeared near the void column volume and at approximately 126 kDa.

salt activation by showing that bile salts do not induce dimerisation at both submicellar and micellar concentrations. Thus, protein dimerisation is not an obligatory component of the bile salt-activated pathway. An additional role for the glycosylated C-terminal domain is also demonstrated in preventing non-specific intermolecular hydrophobic interactions.

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