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THE CATALYTIC CENTER OF LECITHIN:CHOLESTEROL ACYLTRANSFERASE: ISOLATION AND SEQUENCE OF DIISOPROPYL FLUOROPHOSPHATE-LABELED PEPTIDES

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Lecithin:cholesterol acyltransferase (LCAT) was purified from hog plasma and subsequently reacted with [^{3}H]-Diisopropyl fluorophosphate (DFP). The labeled enzyme was digested with pepsin and the peptides separated by high performance liquid chromatography (HPLC). Two radioactive peptides were isolated, subjected to automated amino acid sequencing and yielded the following data: A) Ile-Ser-Leu-Gly-Ala-Pro-Trp-Gly-Gly-Ser, and B) Tyr-Ile-Phe-Asp-x-Gly-Phe-Pro-Tyr-x-Asp-Pro-Val. Both of these sequences represent very highly conserved regions of the enzyme when compared to the sequence of human LCAT. Peptide (A) is considered to represent the catalytic center of LCAT based on comparisons with data reported in the literature.

Lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) is an enzyme that esterifies the free cholesterol transported in plasma lipoproteins (1). As a result LCAT plays a key role in plasma cholesterol homeostasis by maintaining a free cholesterol gradient between plasma membranes and circulating lipoproteins (2) and facilitating the transport of excess peripheral cholesterol to the liver (1). LCAT is a lipolytic enzyme that catalyzes phospholipase and acyltransferase reactions (3). Jauhiainen and Dolphin have recently shown that these reactions, catalyzed by LCAT are sensitive to specific inhibitors: 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) and diisopropylfluorophosphate (DFP), respectively (4). As many other enzymes possessing a serine type active site (5), LCAT is completely inactivated upon treatment with DFP (6). Based on sequence homology data, McLean *et al.* (7) have shown that the interfacial hexapeptide found in porcine pancretic lipase having the primary structure: Ile-Gly-His-Ser-Leu-Gly was also present in human LCAT. Maraganore and Heinrikson (8) have recently suggested that another region of the LCAT molecule may be the site of the active serine residue.

In this communication, we offer evidence that the proposal of Maraganore and Heinrikson is likely to be correct based on our amino acid sequence analyses of peptides bearing the DFP reacting residues in LCAT.

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MATERIALS AND METHODS

Purification of LCAT

LCAT was isolated in homogeneous form from hog plasma according to a previously published procedure involving sequential steps of phenyl-Sepharose, DEAE-Cellulose and hydroxylapatite chromatography. The enzyme preparation obtained by this procedure is purified 30,000 fold, and reasonably stable upon storage (9).

Labeling of the Catalytic Center of LCAT with [^{3}H]-DFP and Digestion of the Labeled Enzyme with Pepsin

LCAT (0.64 mg) was reacted with 0.2 mCi of [^{3}H]-DFP (sp. act. 30 Ci/mMole) in 1.0 mM sodium phosphate, pH 7.4 for 30 min. at 24°C. The excess reagent was removed by dialysis against the same buffer, and the enzyme was lyophilized. The DIP-LCAT was extensively dialysed against 0.1 M sodium acetate, pH 3.0, and an aliquot of pepsin was added. The final concentration of LCAT was 0.43 mg/ml, and the amount of pepsin was adjusted to give a molar ratio of LCAT/pepsin = 10/1. The enzymatic digestion was carried out for 30 min at 37°C. The peptic digest of DIP-LCAT was lyophilized, dissolved in 300 µl of 0.1% trifluoroacetic acid (TFA), and seperated by HPLC (Figure 1).

RESULTS AND DISCUSSION

Isolation and Characterization of the Peptide Fragments Bearing the DFP Reacting Serine Residues

The enzyme preparation labeled with [^{3}H]-DFP contained a sufficient amount of radioactivity to indicate incorporation of approximately 1 mole of DFP per mole of LCAT. Similar observations have been observed by Jauhiainen and Dolphin for the human enzyme (4). Fig. 1 shows the HPLC

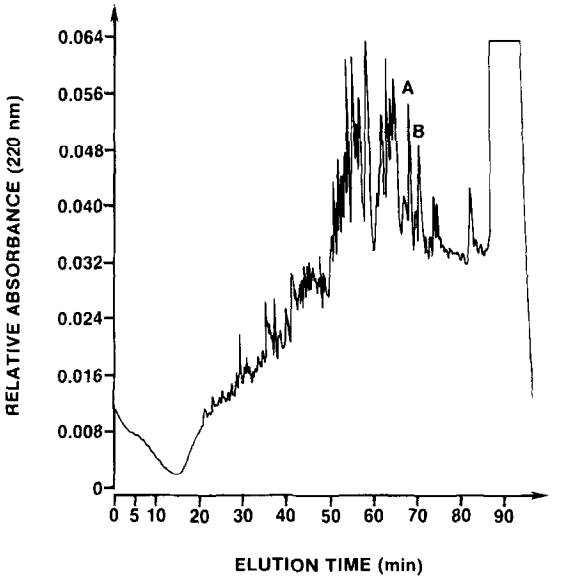

Figure 1. Reverse-phase HPLC separation of peptides derived from peptic digestion of [^{3}H]-DIP-LCAT. The enzyme was labeled with [^{3}H]-DFP and subjected to peptic digestion. The resulting digest was lyophilized, dissolved in 0.1 % aqueous TFA, fractionated by reverse-phase HPLC (C_{18}-column, 4.6 x 250 mm, 5 µm bead size, Bio-Rad; equilibrated with 0.1 % (v/v) aqueous TFA). The peptides were eluted using a linear gradient of 100% Buffer-A to 40 % Buffer-A, 60 % Buffer-B in 90 min. (Buffer A: 0.1% TFA in water, Buffer B: 0.1% TFA in acetonitrile, 1 ml/min flow rate at room temperature, monitored at 220 nm). Fractions were collected manually. An aliquot of each fraction was used to determine the radioactivity. Peaks labeled A & B had the highest radioactivity and were subjected to sequence analysis (Fig. 2).

pattern of the peptic digest of hog LCAT. The pooled fractions under peaks A & B contained a significant amount of radioactivity and were lyophilized prior to sequence analysis. Edman degradation of these peptides using an automated gas phase sequencer (Applied Biosystems Model 470A with on line PTH Analyzer) gave the following sequences:

peptide A: Ile-Ser-Leu-Gly-Ala-Pro-Trp-Gly-Gly-Ser
peptide B: Tyr-Ile-Phe-Asp-x-Gly-Phe-Pro-Tyr-x-Asp-Pro-Val

Fig. 2 shows the yields of amino acid residues per cycle for these peptides. Sequencer program 03RPTH was routinely used to operate the automated sequencer. Accordingly, in this program phenylisothiocyanate (PITC) coupling to N-terminal amino acids and cleavage of the anilinothiazolinone derivatives (ATZ-AA) were carried out at 45°C; the conversion of ATZ-AA into phenylthiohydantoins (PTH-AA) was performed at 55°C. In order to improve the yield and identification of the heat labile serine and threonine residues, sequencing was repeated with program 03RSER in which temperatures were decreased by 5°C. The yield of the first cycle (taken as 100%) was usually high since this residue was subjected to two cycles of PITC-coupling. Low yields of serine are attributed to formation of dehydroalanine and the dithiothreitol (DTT) adduct of PTH-Ser. The data on DTT-PTH-Ser was not included in the calculations but was instrumental in the identification of the serine residues. The low histidine values often observed in this system are presumably due to the low yields of extraction of ATZ-His from the polybrene matrix.

The sequence data shown in Figure 2 were deduced from triplicate runs of peptide A and from a single run of peptide B. Both peptides are highly homologous with corresponding sequences of human LCAT (Fig. 3). The sequence shown in Fig. 2A appears to contain the active site serine. This peptide is 100% homologous with residues 207-216 in human LCAT and also shows a great deal of homology with the active-site peptides of bovine milk lipoprotein lipase (11) and porcine

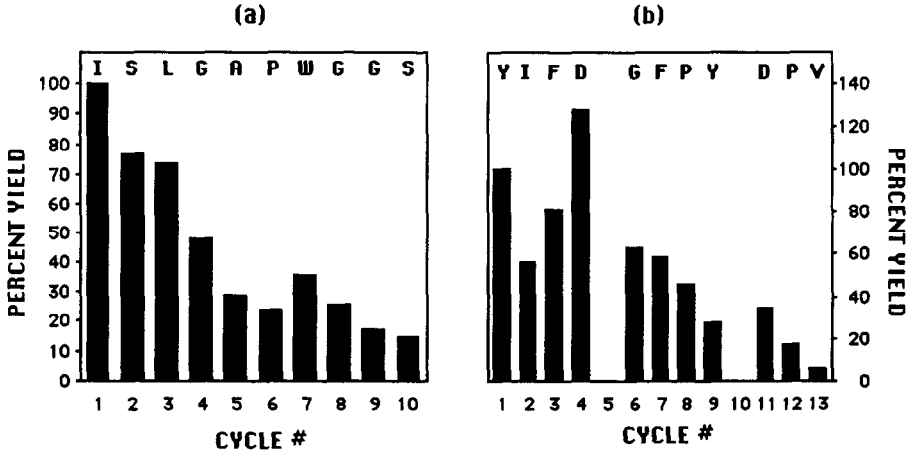

Figure 2. N-terminal sequence analysis by automated Edman degradation of the [^{3}H]-DFP labeled peptic peptides from hog LCAT. Fractions from the HPLC seperation corresponding to [^{3}H]-DIP-peptides (peaks A & B in Fig. 1) were analyzed on Applied Biosystems model 470A gas phase sequencer. Single letter codes of amino acids obtained for each cycle are shown.

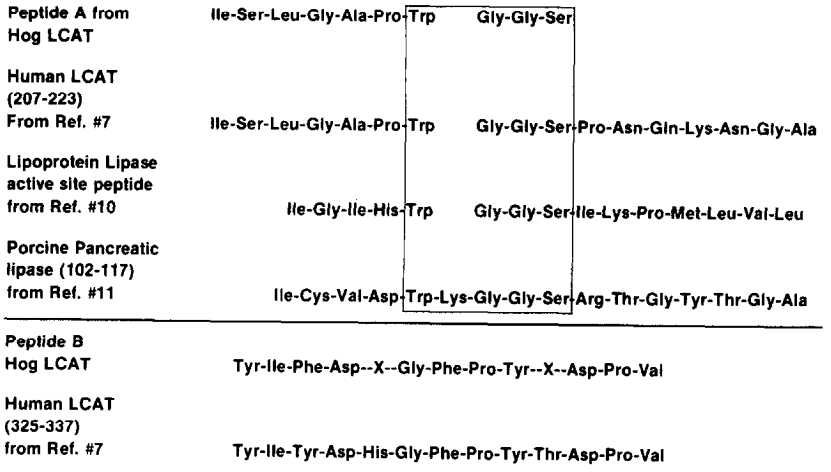

Figure 3. Comparison of the sequences surrounding DFP reacting residues in hog LCAT with similar sequeces found in other lipolytic enzymes.

pancreatic lipase (11) [Fig. 3]. These data support the proposal of Maraganore and Heinrikson (8) who suggested that the segment containing residues 209-223 includes the catalytic serine of human LCAT. Maraganore and Heinrikson further proposed that the so called "interfacial hexapeptide" (Ile-Gly-His-Ser-Leu-Gly) which is completely preserved in pancreatic lipase, lipoprotein lipase and LCAT, may serve as a binding site for macromolecular substrates (8).

Although the precise roles of these respective serine residues in the LCAT mechanism remain to be resolved, our data strongly suggests that the catalytic serine is indeed at the location proposed by Maraganore and Heinrikson (8). The other DFP reacting peptide (Fig. 2B), contains three tyrosine residues and one threonine. In addition there are two unidentified residues that could serve as potential reactive sites for DFP. This peptide is likely to represent a non-specific but highly exposed DFP reacting amino acid side chain in LCAT and the assignment of its role in the LCAT mechanism (if any) must await further investigation.

ACKNOWLEDGEMENTS

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