IDENTIFICATION OF THE LIPID-BINDING CYANOGEN BROMIDE FRAGMENT FROM THE CYSTINE-CONTAINING HIGH DENSITY APOLIPOPROTEIN, APOLP-GLN-II

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SUMMARY:

ApoLP-Gln-II is one of the major protein constituents of human plasma high density lipoproteins (HDL). This protein and its two CNBr fragments, C-III (carboxyl-terminal) and C-IV (amino-terminal) were tested for ability to bind phosphatidyl choline by the formation of lipid-protein complexes of density 1.063 to 1.210 gm/ml, by changes induced in circular dichroism, and by the inhibition of the reactivation of delipidated mitochondrial β-hydroxybutyric dehydrogenase. In all three of these experimental procedures, C-III but not C-IV retained the ability to bind phosphatidyl choline. These findings suggest that the phospholipid binding site(s) of apoLP-Gln-II may be localized in the carboxyl-terminal portion of the molecule.

INTRODUCTION:

The plasma high density lipoproteins (HDL) provide an excellent model for studying phospholipid-protein interactions. HDL contain two major protein moieties, designated by their carboxyl-terminal amino acids as apoLP-Gln-I# and apoLP-Gln-II (1-3), which have been purified and extensively characterized in several laboratories. The lipid-free apolipoproteins are readily soluble in aqueous media and, when sonified with HDL-lipids, reform lipidated complexes which are very similar to the parent molecules (4-7). The complete amino acid sequence of apoLP-Gln-II has recently been published (8). ApoLP-Gln-II contains two identical

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#ApoLP-Gln-I is identical to R-Thr (1), apoA-I (2), and fraction III (3) used in other systems of nomenclature. ApoLP-Gln-II is synonymous with R-Gln (1), apoA-II (2), and fraction IV (3).

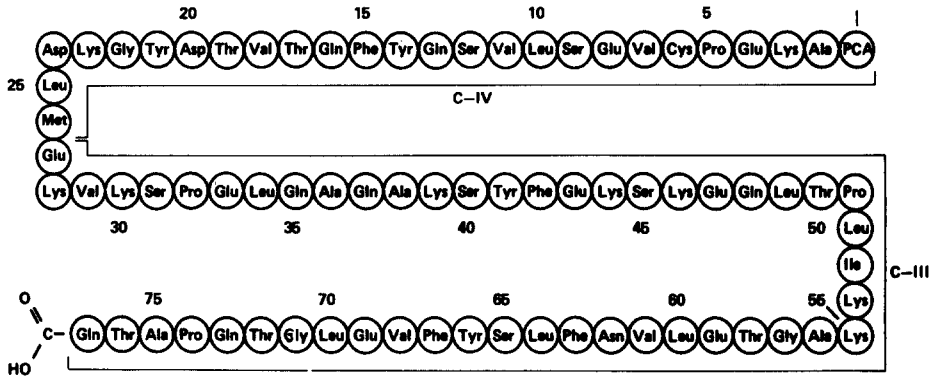

FIGURE 1: The amino acid sequence of apoLP-Gln-II (as determined by Brewer, Lux, Ronan and John[8]) showing the alignment of peptides C-III (C-terminal) and C-IV (N-terminal). ApoLP-Gln-II has been shown to contain two of the polypeptide chains shown above connected by a single disulfide bond at position 6 (9,10). Each chain contains a single methionine residue at position 26. PCA is pyrrolidone carboxylic acid (pyroglutamic acid).

polypeptide chains of 77 amino acids, linked by a disulfide bond (Figure 1). Each chain has a methionine at residue 26 and a carboxyl-terminal glutamine. Cleavage of this protein with CNBr yields two peptides, C-III (carboxyl-terminal) and C-IV (amino-terminal). Full details for the preparation and isolation of these fragments are published elsewhere (9,10). We now report evidence to suggest that the specificity for the binding of phosphatidyl choline may reside in C-III.

METHODS OF PROCEDURE:

HDL were prepared from human plasma by ultracentrifugal flotation between densities 1.063 and 1.210 gm/ml (11) and were delipidated with chloroform-methanol (2:1,v:v) (9) or ethanol-diethyl ether (1:3,v:v) (12). ApoLP-Gln-II was isolated by chromatography of apoHDL on DEAE-cellulose in Tris-HCl, pH 8.0, containing 8 M urea (1,9). The two CNBr peptides of apoLP-Gln-II were prepared in 70% formic acid using a 500:1 ratio of CNBr to methionine and were separated by gel filtration on Sephadex G-75 (Superfine) in 1 M propionic acid (Lux, S.E., K.M.John, R.L.Jackson, S. Fleischer and A.M.Gotto, manuscript in preparation) or as described in detail elsewhere (9,10). The arrangement of these two peptides is shown in Figure 1.

The method for relipidation of apoLP-Gln-II and for examining the relipidation of its CNBr fragments was by incubation with sonically prepared dispersions of phosphatidyl choline, followed by ultracentrifugal flotation in salt solutions (4,7). Egg yolk phosphatidyl choline was purchased from Pierce, and beef heart phosphatidyl choline was prepared by the method of Fleischer and Fleischer (13). Circular dichroism was measured at 23° C under conditions previously described for the study of apoLP-Gln-II (7). Protein concentration was determined by the Lowry procedure (14).

A qualitative assessment of the binding of phosphatidyl choline by apoLP-Gln-II and its fragments was made by measuring the inhibition of the reactivation of delipidated mitochondrial β-hydroxybutyrate dehydrogenase. The apodehydrogenase has an absolute requirement for phosphatidyl choline for enzymic activity. Binding of phosphatidyl choline by apoLP-Gln-II or its fragments results in a decrease in enzymic activity. The magnitude of the decrease is a measure of the relative affinity of the protein or its fragments for the phospholipid. The apodehydrogenase was prepared from beef heart mitochondria by a modification of the procedure of Fleischer et al (15). The assay procedure of Fleischer et al was employed (16). No detergent was present either during the preparation of the apodehydrogenase or during the enzyme assay. The apolipoprotein or its CNBr fragment was incubated for 10 min at 38° C in a reaction mixture containing 3 to 10 μgm of egg or beef heart lecithin, 0.3 μmole of dithiothreitol, NAD (at a concentration to give A_{340} of 0.5) and was buffered with Tris-HCl, pH 8.1. The final Tris buffer concentration was 0.1 M and the total volume of the reaction mixture was 0.90 ml. After 10 min β-hydroxybutyrate apodehydrogenase was added and the mixture was incubated an additional 15 min at 38° C. Twenty μmoles of sodium DL-β-hydroxybutyrate were then added to give a total volume of 1.0 ml and absorbancy changes at 340 nm were measured. A standard curve was run each day in which the activity of the apodehydrogenase was measured at varied concentrations of phosphatidyl choline. In the assays to test lipid-binding,

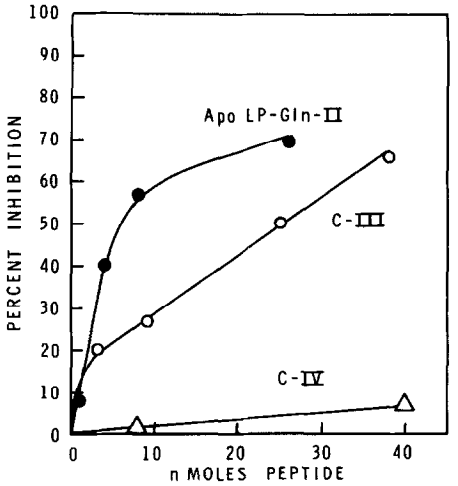

FIGURE 2: Inhibition of the reactivation of mitochondrial β-hydroxybutyrate apodehydrogenase by apoLP-Gln-II (●—●),CNBr fragment C-III (O—O)and CNBr fragment C-IV (Δ—Δ). Please consult the text for details.

a quantity of phosphatidyl choline was used to give one-half of the maximal activity of the dehydrogenase.

RESULTS:

The two CNBr fragments from apoLP-Gln-II, C-III and C-IV (see Fig. 1) were tested for their ability to bind phosphatidyl choline and were compared with the intact protein from which they were prepared. As a basis for comparison, the starting protein, apoLP-Gln-II, with an intact disulfide bond, was employed. No significant differences were observed in lipid-binding activity between apoLP-Gln-II before or after reduction and aminoethylation. The interaction between the apolipoprotein and its fragments with phosphatidyl choline was studied in three different experimental systems with three different preparations of the CNBr fragments C-III and C-IV.

1. Ultracentrifugal flotation of lipid-protein complexes. The binding of phosphatidyl choline was tested by the formation of protein- or peptide-phosphatidy choline complexes which floated between densities 1.063 and 1.210 gm/ml. When an equal weight of apoprotein or peptide fragment was incubated with phosphatidyl choline, 65% of the added apoLP-Gln-II was isolated as a lipid-protein complex

of density less than 1.210 gm/ml. Under identical conditions, 50% of the added C-III but only 15% of the C-IV bound sufficient phospholipid to float in the HDL density range.

2. Circular dichroism. C-III and C-IV had circular dichroism (CD) spectra indicating predominantly disordered secondary structure. On the other hand, apoLP-Gln-II contained approximately 35% α-helix as previously described (7). Relipidation of apoLP-Gln-II with phosphatidyl choline increased the helicity, changing the mean residue ellipticity at 221 nm, $[\theta]_{221}$, from -14,000 to -16,000 deg cm^2 per dmole. Incubation of C-III with phosphatidyl choline resulted in a marked increase in α-helix content as indicated by a change in $[\theta]_{221}$ from -4,000 to -12,000. Incubation of C-IV under identical conditions did not significantly change $[\theta]_{221}$, which was -1,000 both before and after exposure to phospholipid.

3. Inhibition of reactivation of β-hydroxybutyrate apodehydrogenase. ApoLP-Gln-II was a very potent inhibitor of the reactivation of delipidated β-hydroxybutyric dehydrogenase prepared from mitochondria. While the relative affinity of C-III for phosphatidyl choline was less than that of apoLP-Gln-II, this CNBr fragment did exhibit a significant binding of phosphatidyl choline (Fig. 2). On the other hand, C-IV exhibited little or no binding of phosphatidyl choline in the assay; similar results were obtained with lysozyme and ribonuclease.

DISCUSSION:

Because its primary structure is completely known (8) (Fig. 1), apoLP-Gln-II is of particular interest as a model for studying lipid-protein interactions. The aim of such studies is to identify and "map" the lipid binding site(s) in order to determine the specific interactions which result in phospholipid binding. One approach to this problem is to assay the lipid-binding ability of various proteolytic fragments of apoLP-Gln-II in the expectation that regions of the molecule containing such sites might be isolated. Cyanogen bromide cleavage of apoLP-Gln-II was chosen in order to create two large fragments of the protein. The enzymatic inhibition assay of Fleischer et al (16) was particularly useful because of limited quantities of the purified peptides. Only micrograms of

each peptide were required to substantially inhibit in the assay system. In additi the method was rapid, well suited for obtaining a quick, qualitative assessment of phospholipid binding in multiple samples, and provided a measure of binding avidity as well as capacity. Its principal disadvantages were that only phosphatidy choline could be used, that the reaction conditions could not be varied widely, and that results from different experiments could not be compared easily because of variations in the activity of the enzyme preparations. Since phosphatidyl choline is the major phospholipid constituent of HDL, the specificity of the dehydrogenase was not a disadvantage for purposes of our experiments.

The results of the present study provide evidence for the preferential binding of phosphatidyl choline by the carboxyl-terminal two-thirds of apoLP-Gln-II. The ability to bind phosphatidyl choline is not generally shared by other soluble proteins. A significant interaction between C-III and phosphatidyl choline was demonstrated by three independent experimental techniques, while no interaction could be shown under identical conditions with C-IV. The present study cannot absolutely exclude the possibility that the native lipid-binding site(s) of apoLP-Gln-II was disrupted by the CNBr cleavage and that the lipid-binding properties exhibited by C-III were artifactual. Other studies are needed in which the lipid-binding site(s) is identified in the intact molecule. Affinity labeling techniques in particular, hold promise of pinpointing particular residues at or adjoining lipid-binding sites. Such studies are currently in progress.

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