Human Plasma High Density Lipoprotein

INTERACTION OF THE CYANOGEN BROMIDE FRAGMENTS FROM APOLIPROTEIN GLUTAMINE II (A-II) WITH PHOSPHATIDYLCHOLINE*

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SUMMARY

Apolipoprotein-glutamine-II (apoLP-Gln-II) is one of the two major protein constituents of human plasma high density lipoproteins (HDL). ApoLP-Gln-II contains two identical chains of 77 amino acids each, which are linked by a single disulfide bond at residue 6. Each chain contains a single methionine at position 26. In the present study we have examined the phospholipid binding properties of apoLP-Gln-II, of the NH₂-terminal cyanogen bromide fragment both in the reduced form (CNBr IV, residues 1 to 26) and with the disulfide bond intact (CNBr IV)₂, of the COOH-terminal cyanogen bromide fragment (CNBr III, residues 27 to 77) and of performic acid-oxidized apoLP-Gln-II. The binding of phosphatidylcholine was monitored by the inhibition of the reactivation of delipidated α-hydroxybutyrate dehydrogenase from beef heart mitochondria and by the formation of phospholipid-protein complexes which were isolated by density gradient ultracentrifugation in sucrose. The circular dichroism and immunochemical properties of these fragments and derivatives of apoLP-Gln-II were studied both in the presence and absence of phosphatidylcholine. The results may be summarized as follows. (a) ApoLP-Gln-II and its performic acid-oxidized derivative were potent inhibitors of the reactivation of the dehydrogenase. One microgram of these preparations gave approximately 50% inhibition. The COOH-terminal fragment, CNBr III, also inhibited reactivation of the dehydrogenase at low concentrations, but was less effective than the intact protein. Both CNBr IV and (CNBr IV)₂ failed to inhibit reactivation of the dehydrogenase. (b) ApoLP-Gln-II, performic acid-oxidized apoLP-Gln-II and CNBr III formed complexes with phosphatidylcholine which were isolated by density gradient ultracentrifugation in sucrose (d 1.063 to 1.210). These isolated complexes contained, respectively, 1.16, 1.56, and 2.00 mg of phosphatidylcholine per mg of protein or peptide. When (CNBr IV)₂ was reconstituted with phosphatidylcholine and subjected to ultracentrifugation under identical conditions, the isolated peptide contained only 0.08 mg of phospholipid per mg of peptide. (c) Reconstitution with phosphatidylcholine resulted in an apparent increase in α helical content (as judged by an increase in 0.222 nm in the circular dichroism spectrum). The magnitude of the changes were as follows: 49% to 64% for apoLP-Gln-II, 32 to 57% for performic acid-oxidized apoLP-Gln-II, and 32 to 44% for CNBr III. There was no significant change in the CD spectra of (CNBr IV)₂ detected in the presence of phosphatidylcholine. (d) Performic acid oxidation of apoLP-Gln-II did not alter the qualitative immunochemical precipitin lines when this antigen was tested against rabbit antisera prepared against apoLP-Gln-II. The same antiserum reacted with CNBr III and (CNBr IV)₂, but formed lines of only partial identity between the fragments or between each fragment and apoLP-Gln-II. Rabbit antisera to CNBr III formed precipitin lines of complete identity between this fragment, apoLP-Gln-II, and performic acid-oxidized apoLP-Gln-II. No differences were noted after reconstitution with phosphatidylcholine. Anti-CNBr III did not form detectable precipitin lines with (CNBr IV)₂.

By the tests we have employed, we conclude that CNBr III or the COOH-terminal two-thirds of apoLP-Gln-II exhibits preferential interaction with phosphatidylcholine, although to a lesser extent than does the intact protein. The NH₂-terminal fragment did not show a significant interaction with phosphatidylcholine whether the disulfide linkage was intact.

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or not. Modification of the disulfide linkage or of the methionine at residue 26 did not alter the qualitative immunoprecipitin test with the antisera employed.

Despite the widespread interest in the structure of plasma and membrane lipoprotein complexes, little is known of the molecular details of lipid-protein interactions. We have recently embarked on a series of studies designed to identify and “map” the phospholipid binding site or sites in apolipoprotein-Gln-I and apolipoprotein-Gln-II, the two major protein moieties of human plasma high density lipoproteins (HDL). These two proteins, designated by their COOH-terminal amino acids, have been purified and extensively characterized in several laboratories. The physicochemical properties of the HDL-proteins have been discussed in several recent reviews (1-4). The complete amino acid sequence of apoLP-Gln-II has recently been published (5). The sequence of the first 40 residues of apoLP-Gln-II has also been reported (3). Both apoLP-Gln-I and apoLP-Gln-II are readily soluble in aqueous media and can be recombined with HDL-lipids to form complexes similar in several properties to the parent HDL (6-11).

ApoLP-Gln-II was chosen for particular scrutiny because recombination and degradation studies of HDL suggest that it may have a greater affinity for lipid than apoLP-Gln-I (12, 13). ApoLP-Gln-II consists of two identical polypeptide chains of 77 residues, with NH₂-terminal pyrrolidone carboxylic acid and COOH-terminal glutamine (Fig. 1). The two chains are linked by a disulfide bridge between their sixth residues. Each chain contains a single residue of methionine at position 20. Cyanogen bromide cleavage of reduced and aminoethylated apoLP-Gln-II yields an NH₂-terminal peptide containing 26 amino acids (CNBr IV) and a COOH-terminal peptide containing 51 residues (CNBr III) (14). Results presented in preliminary form (15) suggested that CNBr III retains the ability to bind phospholipid even though its secondary structure is altered compared to apoLP-Gln-II; the reduced, carboxymethylated NH₂-terminal fragment (CNBr IV) did not bind lipid.

In the present communication we describe the lipid-binding studies of CNBr IV with the disulfide intact (CNBr IV)². We have also isolated CNBr III after reconstitution with phosphatidylcholine using density gradient ultracentrifugation in sucrose and extensively characterized in several laboratories. The physicochemical properties of the isolated complex were compared to those of (CNBr IV)², apoLP-Gln-II and performic acid-oxidized apoLP-Gln-II, both with and without phospholipid. The lipid-binding properties of apoLP-Gln-II are described.

**EXPERIMENTAL PROCEDURE**

**Preparation of ApoLP-Gln-II and Derivatives—HDL** were isolated ultracentrifugally from normal fasting donors between density 1.063 and 1.210 g per ml (14), and were delipidated with diethylether-ethanol (3:1). The lipid-free proteins or apolipoproteins were chromatographed on Sephadex G-150 in 5.4 M urea (14) or on DEAE-cellulose (16). Homogeneity of apoLP-Gln-II (Fraction IV of Edelstein et al. (17)) was established by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (18) or urea (19); by immunoprecipitin reaction with antisera prepared against apoLP-Gln-II and apoHDL; and by amino acid composition. ApoLP-Gln-II was reduced and aminoethylated as previously described (14) and was oxidized with performic acid by the method of Hirs (20). The reduced and aminoethylated...
protein contained 0.9 mole of aminoethylcysteine per 77 residues of amino acids.

**Isolation of Cyanogen Bromide Fragments—ApoLP-Gln-II (20 mg) in 70% formic acid was treated with a 500-molar excess of cyanogen bromide for 24 hours at 23°C.** The lyophilized digest was dissolved in 2.0 ml of 0.1 M Tris-HCl, 8 M urea, and the pH adjusted to 8.6. To cleave the disulfide bond in the NH₂-terminal fragment (Fig. 1), 0.25 ml of β-mercaptoethanol was added to the digest. After incubation for 4 hours at 37°C, the fragments were subjected to chromatography on Sephadex G-50. The column (1.6 x 200 cm) was equilibrated with 0.1 M Tris-HCl, pH 8.0, containing 5.4 M urea and 1 mM β-mercaptoethanol, and was operated at a flow rate of 25 ml per hour. The fractions corresponding to CNBr III and CNBr IV (Fig. 2) were desalted and lyophilized. Gel filtration on Sephadex G-50 of Zone IV from A. CNBr IV was subjected to reoxidation as described in the text. Redetermination of oxidized CNBr IV was performed in exactly the same manner as described in A with the exception that β-mercaptoethanol was eliminated from the buffer.

**Isolation of Cyanogen Bromide Fragments—**The apoprotein (20 mg) was treated with cyanogen bromide and the digest subjected to Sephadex G-50 chromatography on a column (1.6 x 200 cm). The fragments were eluted with 0.1 M Tris-HCl, pH 8.0, containing 5.4 M urea and 1 mM β-mercaptoethanol. The flow rate was 25 ml per hour and 4-ml fractions were collected. Zones corresponding to the protein contained 0.9 mole of aminoethylcysteine per 77 residues of amino acids.

**Isolation of Protein-phosphatidylcholine Complexes by Density Gradient Ultracentrifugation Reconstitution of apoLP-Gln II and the CNBr fragments with phospholipid was carried out as previously described (10), using purified phosphatidylcholine from egg yolk (26, 27).** Dispersions were prepared by sonication of the phosphatidylcholine for 1 hour at 15°C under nitrogen (28). As determined by thin layer chromatography, the preparation did not contain lysolecithin or phosphatidic acid. In the present study, CNBr III represents residues 27 to 77 of apoLP-Gln-II and CNBr IV residues 1 to 26. (CNBr IV)² represents CNBr IV with the disulfide restored.

**β-Hydroxybutyrate dehydrogenase from beef heart mitochondria is a phospholipid-requiring enzyme (21). When prepared in the lipid-free state, the dehydrogenase has an absolute requirement for phospholipid for reactivation.** The binding assay employed in our experiments depended on the competition of apoLP-Gln-II or its derivatives or fragments for available phosphatidylcholine. To the extent that phospholipid was unavailable to the dehydrogenase, the observed reaction rate was reduced (22). The apodehydrogenase was isolated from beef heart mitochondria in soluble form by modification (23) of the procedure of Rouser and Fleischer (24). The soluble enzyme preparation was released from bovine heart mitochondria by treatment with phospholipase A and was purified through the stage of the acid precipitation step. As isolated, the enzyme contained no detergent and had an absolute and specific requirement for phospholipid. The phosphatidylcholine used in the assay was purified from bovine heart (25) and was dispersed in 20 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, as previously described (29).

The assay was carried out by preincubating the apoLP-Gln-II, RaapoLP-Gln-II, or its CNBr fragments for 10 min at 38°C in a reaction mixture of 0.9 ml containing phosphatidylcholine (10 to 28 μg by weight of lipid), 50 μmoles of EDTA, 0.3 μmole of dithiothreitol, 2 μmoles of DPN, and 0.6% ethanol (v/v). After 10 min, the apodehydrogenase (9 to 23 μg) was added and the mixture incubated 15 min at 38°C. The enzymic reaction was initiated by addition of sodium β-hydroxybutyrate, 20 μmoles, to give a total volume of 1.0 ml and absorbance changes at 340 nm were measured. A phospholipid concentration curve for the dehydrogenase was run daily. A quantity of phosphatidylcholine was used in the competition binding studies which gave one-half of the maximal activity of the dehydrogenase. This amount of lipid was approximately 10 to 28 μg by weight of beef heart phosphatidylcholine and is about one-fifth the amount of lipid required for maximal activity.

**Isolation of Protein-phosphatidylcholine Complexes by Density Gradient Ultracentrifugation** Reconstitution of apoLP-Gln II and the CNBr fragments with phospholipid was carried out as previously described (10), using purified phosphatidylcholine from egg yolk (26, 27). Dispersions were prepared by sonication of the phosphatidylcholine for 1 hour at 15°C under nitrogen (28). As determined by thin layer chromatography, the preparation did not contain lyssolecithin or phosphatidic acid. A volume (0.4 ml) of the phospholipid dispersion (32 mg per ml) in 1 mM Tris-HCl, 0.1 M NaCl, and 10 mM EDTA, pH 7.4, was added to apoLP-Gln-II (10 μg), performic acid-oxidized apoLP-Gln-II (1.13 mg), CNBr III (0.58 mg), or CNBr IV² (1.6 mg). Each sample was then subjected to sonication for 30 s at 0°C with a Crown sonicator equipped with a microtip probe at a minimum power setting. After sonication, the pH of each sample was 7.4. The samples were then incubated at 23°C for 30 min. The protein-phospholipid complexes were isolated by ultracentrifugal flotation in gradients of sucrose (d 1.063 to 1.210 g per ml) as recently described (28). The gradients were prepared in 5-ml nitrocellulose tubes and equilibrated overnight at 4°C; 0.3 ml of each sample was applied to the top of the gradient. The samples were subjected to ultracentrifugation at 7°C in a Beckman SW 50.1 rotor at 30,000 rpm (248,000 x g). After 90 hours the gradients were fractionated and 20-drop fractions collected. Protein was...
isolated protein-phosphatidylcholine complexes were analyzed for phospholipid by the method of Bartlett (30) and for protein by amino acid analysis.

Circular Dichroism (CD) Measurements—The binding of phosphatidylcholine by apoLP-Gln-II has been shown to be attended by changes in the far ultraviolet CD spectrum of the protein (10). There is an increase in the negative ellipticity at 222 nm, which based on comparisons with polypeptides and proteins of known conformation, is consistent with an increase in α helical structure (10). A sonified dispersion of egg phosphatidylcholine, prepared as above, was added in increments to a known quantity of the protein or peptide. After each increment of phospholipid, the mixture was sonicated at 4°C for 30 s at minimum power. The far ultraviolet CD spectrum was then recorded at 25°C using a Cary 61 spectropolarimeter, as previously described (10). Additions of phospholipid were continued until no further change was observed in the CD spectra. The reproducibility of measurements was within 3%. The instrument was calibrated with α-camphorsulfonic acid. The α helical content was estimated from the CD spectra as described by Greenfield and Fasman (31).

Other Procedures—Rabbit antisera were prepared against apoHDL, apoLP-Gln-II, and CNBr III by previously described methods (32). Amino acid analyses were performed on Beckman models 117 or 119 amino acid analyzers.

## RESULTS

Establishment of Purity of ApoLP-Gln-II—The homogeneity of apoLP-Gln-II was established in several ways after isolation from apoHDL by chromatography on Sephadex G-150. It migrated as a single component upon polyacrylamide gel electrophoresis in sodium dodecyl sulfate or in urea; it formed a single immunoprecipitin line with antiserum prepared against apoLP-Gln-II, and its amino acid composition was consistent with the published amino acid sequence (5) (Table I).

Performic Acid-oxidized Derivative of ApoLP-Gln-II—Performic acid oxidation converted apoLP-Gln-II from the dimer to the monomeric form and resulted in the formation of 0.9 mole of cysteic acid and 1.0 mole of methionine sulfone per 77 residues.

Cyanogen Bromide Fragments of ApoLP-Gln-II—Cyanogen bromide cleavage of apoLP-Gln-II is expected to yield an NH2-terminal fragment with 26 residues and a COOH-terminal fragment with 51 residues (Fig. 1). Chromatography of the cyanogen bromide digest of apoLP-Gln-II on Sephadex G-50 in β-mercaptoethanol resolved four fragments (Fig. 2A). The first fraction eluted at the void volume of the column and was not characterized in the present study. The second fraction had an amino acid composition similar to apoLP-Gln-II, except that the methionine content was low and methionine sulfone was present. This finding is consistent with the results of Lux et al. (16, 33) and suggests that Fraction II represents primarily apoLP-Gln-II which has resisted cleavage with cyanogen bromide because of methionine oxidation. The third and fourth fractions contained unique peptides which represented, respectively, the COOH-terminal (CNBr III, 51 residues) and NH2-terminal (CNBr IV, 26 residues) fragments of apoLP-Gln-II. CNBr III and IV completely accounted for the amino acid composition of apoLP-Gln-II (Table I). CNBr III contained no half-cystine, homoserine, homoserine lactone, or methionine. CNBr IV (reduced) contained homoserine and half-cystine.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>ApoLP-Gln-II</th>
<th>CNBr III</th>
<th>CNBr IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>8.5 (9)</td>
<td>6.0 (7)</td>
<td>2.1 (3)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>3.1 (3)</td>
<td>1.1 (1)</td>
<td>2.0 (2)</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.5 (6)</td>
<td>3.8 (4)</td>
<td>1.8 (2)</td>
</tr>
<tr>
<td>Serine</td>
<td>5.5 (6)</td>
<td>5.8 (4)</td>
<td>1.9 (2)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>16.2 (16)</td>
<td>11.1 (11)</td>
<td>5.4 (5)</td>
</tr>
<tr>
<td>Proline</td>
<td>4.0 (4)</td>
<td>2.8 (3)</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.0 (3)</td>
<td>2.1 (2)</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.3 (5)</td>
<td>3.8 (4)</td>
<td>0.9 (1)</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>0.2b (1)</td>
<td>----</td>
<td>0.2b (1)</td>
</tr>
<tr>
<td>Valine</td>
<td>5.6 (6)</td>
<td>2.8 (3)</td>
<td>3.1 (2)</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.7 (1)</td>
<td>----</td>
<td>0.4 (1)c</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.8 (1)</td>
<td>0.8 (1)</td>
<td>----</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.2 (8)</td>
<td>6.3 (6)</td>
<td>2.1 (2)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.8 (5)</td>
<td>1.9 (2)</td>
<td>2.0 (2)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.7 (4)</td>
<td>2.8 (3)</td>
<td>0.9 (1)</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>77</td>
<td>51</td>
<td>26</td>
</tr>
</tbody>
</table>

a Based on the monomer form  

b Partially destroyed during acid hydrolysis  

c As homoserine  

Reduced (Monomeric) and Oxidized Forms of CNBr IV—As isolated, CNBr IV was in the monomeric form with the interchain disulfide reduced. To reform the disulfide bond, the reduced peptide was oxidized with molecular oxygen as described under “Experimental Procedure.” The oxidized (CNBr IV)2 was isolated by chromatography on Sephadex G-50 in the absence of β-mercaptoethanol (Fig. 2B). Since the reoxidized, dimeric (CNBr IV)2 contains 52 amino acid residues, and CNBr III contains 51 residues, it is not feasible to separate mixtures of these substances by gel filtration chromatography (see Fig. 2). Hence, an initial separation of CNBr III was obligatory, prior to the reoxidation of CNBr IV. An examination of the kinetics of disulfide reformation was beyond the scope of the present study.

Inhibition of Reactivation of β-Hydroxybutyrate Apodehydrogenase—ApoLP-Gln-II was a potent inhibitor of the reactivation of β-hydroxybutyrate apodehydrogenase, with 1 μg giving approximately 50% inhibition (Fig. 3). Reduction and alkylation of the disulfide bond did not alter the binding of phosphatidylcholine by apoLP-Gln-II in this assay (Fig. 3), consistent with other recent studies from this laboratory (28). Results with the performic acid-oxidized apoLP-Gln-II were indistinguishable from those with apoLP-Gln-II. The COOH-terminal fragment, CNBr III, exhibited definite binding of phospholipid in this assay system, although at low concentrations it was only one-seventh to one tenth as effective as the uncleaved protein, based on the weight of protein which is necessary for 50% inhibition of β-hydroxybutyrate dehydrogenase. By contrast, the NH2-terminal cyanogen bromide peptide, (CNBr IV)2, had little inhibitory effect (8% inhibition per 6 μg peptide). The results with (CNBr IV)2 were indistinguishable whether the disulfide linkage was reduced or intact.
FIG. 3. Inhibition of the reactivation of mitochondrial \( \beta \)-hydroxybutyrate apodehydrogenase by apoproteins. ApoLP-Gln-II (\( \bullet \bullet \bullet \)) reduced-aminoethylated apoLP-Gln-II (\( \bullet \bullet \bullet \bullet \)), CNBr III (\( \bullet \bullet \bullet \bullet \bullet \bullet \)), and CNBr IV (\( \bullet \bullet \bullet \bullet \bullet \bullet \bullet \bullet \bullet \bullet \bullet \)) were incubated at 38° in the assay mixture containing 9.9 \( \mu \)g of phosphatidylcholine. After 10 min, 9 \( \mu \)g of \( \beta \)-hydroxybutyrate apodehydrogenase were added and the mixture was incubated 15 min at 38°. DL-\( \beta \)-Hydroxybutyrate (20 \( \mu \)moles) was then added and the absorbance increase at 340 nm was recorded. Details of the assay are given under "Experimental Procedure." 

Reconstitution of Apoproteins—ApoLP'-Gln-II, its performic acid-oxidized derivative, and its cyanogen bromide fragments CNBr III and (CNBr IV)\( _z \) were reconstituted with phosphatidylcholine as described under "Experimental Procedure." To insure proper mixing the samples were usually sonicated briefly at 0°, but the results were not significantly altered when the sonication step was omitted. The complexes were isolated by ultracentrifugation (Figs. 4 and 5) in gradients of sucrose of density 1.063 to 1.210 g per ml as described previously (28). The purpose of these experiments was to separate and isolate the protein-phospholipid complexes from unbound protein and phosphatidylcholine. It was not necessary for this purpose to reach equilibrium conditions; nor was it practicable to do so, since it was shown in separate experiments that 200 hours of ultracentrifugation were required for the complex of CNBr III-phosphatidylcholine to come to equilibrium. Therefore, the sucrose density corresponding to a particular fraction (Figs. 4 and 5) does not represent the actual density of the complex at equilibrium. In control experiments, apoLP-Gln-II sedimented to density 1.15 g per ml in the absence of phospholipid (Fig. 4A). Phosphatidylcholine in the absence of added protein was found at the top of the gradient (Fig. 4B). The complex of apoLP-Gln-II-phosphatidylcholine sedimented in the density range of 1.05 to 1.12 g per ml (Fig. 4C). The ratio of phospholipid to protein was not constant throughout the peak; this suggests that complexes with varying degrees of lipidation are formed. The ratio of phospholipid to protein of the pooled complex was 1.16 to 1 by weight (Table II).

In the experiments shown in Fig. 5, the performic acid-oxidized derivative of apoLP-Gln-II also formed a complex with phosphatidylcholine (Fig. 5A) and was isolated at a density of 1.08 g per ml at an average phospholipid to protein ratio of 1.56 to 1 (Table II). It is not known whether the differences between the phospholipid content of the complexes with apoLP-Gln-II and apoLP'-Gln-II plus phosphatidylcholine (Fig. 5B) and (CNBr IV)\( _z \) plus phosphatidylcholine (Fig. 5C) are significant. For the experimental details please refer to Fig. 4.
Phospholipid and protein content were determined as described under "Experimental Procedure." Each sample was dialyzed against 0.05 M Tris-HCl, pH 7.4, and the phospholipid and protein content determined as described under "Experimental Procedure." 

Isolation of protein-phospholipid complexes by density gradient centrifugation

ApoLP-Gln-II (1.10 mg), performic acid-oxidized apoLP-Gln-II (1.13 mg), CNBr III (0.88 mg), and (CNBr IV) (1.60 mg) were recombined with phosphatidylcholine (120 mg) and the complexes isolated by sucrose gradient centrifugation as described in the text. Each sample was dialyzed against 0.05 M Tris-HCl, pH 7.4, and the phospholipid and protein content determined as described under "Experimental Procedure." 

<table>
<thead>
<tr>
<th>Protein or peptide</th>
<th>Protein recovered</th>
<th>Composition</th>
<th>Ratio of PC to protein (by weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoLP-Gln-II</td>
<td>51</td>
<td>576 / 1258</td>
<td>1.36</td>
</tr>
<tr>
<td>Performic acid-oxidized apoLP-Gln-II</td>
<td>42</td>
<td>309 / 1739</td>
<td>1.56</td>
</tr>
<tr>
<td>CNBr III</td>
<td>74</td>
<td>1258 / 100</td>
<td>0.90</td>
</tr>
</tbody>
</table>

a Refers to the complexes corresponding to the bars shown in Fig. 5.

b PC, phosphatidylcholine.

c Its performic acid-oxidized derivative of significance. CNBr-III formed a complex with phosphatidylcholine that was isolated at a density of 1.09 g per ml and which contained an average lipid to protein ratio of 2.00 to 1 (Fig. 5B and Table II). (CNBr IV) was isolated at an average density of 1.15 g per ml under the non-equilibrium conditions (Fig. 5C). The fractions containing the protein were essentially devoid of lipid, the phosphatidylcholine to protein ratio being 0.08 (Table II). Virtually all of the phosphatidylcholine was at the top of the gradient, i.e. in tubes 14 and 15.

A comment should be made about the protein associated with the excess phosphatidylcholine at the top of the gradient. We have found only proteins or fragments of proteins which bind phosphatidylcholine at the top of the gradient. Ribonuclease, lysozyme, and (CNBr IV), all of which do not bind phosphatidylcholine, did not float either with the excess lipid or with lipid elsewhere in the gradient. These findings suggest that there may be two or more mechanisms for binding phosphatidylcholine.

It will be noted that the protein recoveries varied from 41 to 74% of the added protein (Table II). The recovery refers only to the quantity of complex recovered in the major protein peaks from the intermediate density ranges between the top and bottom of the tube. With apoLP-Gln-II, performic acid-oxidized apoLP-Gln-II and CNBr III, there were significant quantities of protein associated with the excess phosphatidylcholine at the top of the gradient. There was no detectable protein at the top of the gradient in the reconstitution experiment with phosphatidylcholine and (CNBr IV) isolated from the density gradient ultracentrifugation experiment. 

**Circular Dichroism Studies**—Performic acid oxidation of apoLP-Gln-II resulted in a significant decrease in the negative ellipticity at 222 nm (Fig. 6A). Estimation of the α helical content of (31) indicated approximate values of 49% for apoLP-Gln-II and of 32% for its performic acid-oxidized derivative. COOH-terminal cyanogen bromide fragment (CNBr III) had both disordered and α helical structures (Fig. 6B); the estimated α helical content was 32%. The spectrum of (CNBr IV) indicated predominantly disordered structure (Fig. 6B). After reconstitution with phospholipid (Table II), significant increases in the magnitude of the negative ellipticity at 222 nm were observed for apoLP-Gln-II, performic acid-oxidized apoLP-Gln-II, (Fig. 6A and CNBr III (Fig. 6B)). ApoLP-Gln-II had an estimated helicity of 64% and the performic acid-oxidized derivative of 57%. Reconstitution of CNBr III with phospholipid increased the estimated helicity from 32% to 44%. In each instance, reconstitution with phosphatidylcholine was attended by significant, directly observable changes in the CD spectra (Fig. 6A and B). In contrast to the results with CNBr III, (CNBr IV) isolated from the density gradient ultracentrifugation experiment showed no significant changes in its CD spectrum (Fig. 6B). In other experiments, apoLP-Gln-II, performic acid-oxidized apoLP-Gln-II and each of the cyanogen bromide fragments were titrated with increasing amounts of phosphatidylcholine as shown in Fig. 7. The addition of phospholipid to apoLP-Gln-II, performic acid-oxidized apoLP-Gln-II and CNBr III produced an increase in the magnitude of the negative ellipticity at 222 nm. In contrast to CNBr III, the NH2-terminal fragment (CNBr IV) produced no alteration in the far ultraviolet CD spectrum; the addition of up to 25.7 mg of phosphatidylcholine caused no significant change in the CD spectrum.

**Immunochemical Properties**—Rabbit antisera prepared against apoLP-Gln-II gave single precipitin lines of complete identity.

The α helical content was calculated from the equation:

\[ \% \text{helix} = \frac{\text{mean residue ellipticity} + 3000}{36,000 + 3,000} \]

\[ \times 10^{-3} \text{ DEGREE CD/MOLE} \]

**Table II**

Isolation of protein-phospholipid complexes by density gradient centrifugation

<table>
<thead>
<tr>
<th>Protein or peptide</th>
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<th>Ratio of PC to protein (by weight)</th>
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<td>ApoLP-Gln-II</td>
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</tr>
<tr>
<td>CNBr III</td>
<td>74 / 100</td>
<td>0.90</td>
</tr>
</tbody>
</table>

\[ \% \text{helix} = \frac{36,000 + 3,000}{\text{mean residue ellipticity}} \]

\[ \times 10^{-3} \text{ DEGREE CD/MOLE} \]

**Fig. 6.** Circular dichroic spectra of apoproteins and phosphatidylcholine-apoprotein complexes. A, apoLP-Gln-II (--); ApoLP-Gln-II plus phosphatidylcholine (---); performic acid-oxidized apoLP-Gln-II (--.--); performic acid-oxidized apoLP-Gln-II plus phosphatidylcholine (---.--); CNBr III (--.--); CNBr III plus phosphatidylcholine (---.--); (CNBr IV) plus phosphatidylcholine (---.--).

**Fig. 7.** Circular dichroic spectra of apoproteins and phosphatidylcholine-apoprotein complexes. A, apoLP-Gln-II (--); ApoLP-Gln-II plus phosphatidylcholine (---); performic acid-oxidized apoLP-Gln-II (--.--); performic acid-oxidized apoLP-Gln-II plus phosphatidylcholine (---.--); CNBr III (--.--); CNBr III plus phosphatidylcholine (---.--); (CNBr IV) plus phosphatidylcholine (---.--).

**Table II**

<table>
<thead>
<tr>
<th>Protein or peptide</th>
<th>Composition</th>
<th>Ratio of PC to protein (by weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoLP-Gln-II</td>
<td>51 / 1258</td>
<td>1.36</td>
</tr>
<tr>
<td>Performic acid-oxidized apoLP-Gln-II</td>
<td>42 / 1739</td>
<td>1.56</td>
</tr>
<tr>
<td>CNBr III</td>
<td>74 / 100</td>
<td>0.90</td>
</tr>
</tbody>
</table>
Fig. 7. Increase in the negative mean residue ellipticity (θ) of apoproteins with the addition of phosphatidylethanolamine. A dispersion of egg phosphatidylethanolamine was added in increments to a known quantity of apoLP-Gln-II (●—●); performic acid-oxidized apoLP-Gln-II (○—○); CNBr III (Δ—Δ); and (CNBr IV)² (■—■). After each addition of lipid, the spectrum was recorded.

between the antibody and the reduced-aminoethylated and performic acid-oxidized apoLP-Gln-II derivatives (Fig. 8A). The antisera also reacted with CNBr III and (CNBr IV)², but formed precipitin lines of only partial identity between the fragments or between each fragment and the uncleaved protein (Fig. 8A). The faint precipitin line between anti-apoLP-Gln-II sera and (CNBr IV)² could not be detected if the peptide was mixed with a dispersion of phosphatidylethanolamine (Fig. 8B). The same antibody preparation formed a precipitin line with CNBr III reconstituted with phosphatidylethanolamine (Fig. 8B). This line showed partial identity with apoLP-Gln-II and with apoLP-Gln-II reconstituted with phospholipid (Fig. 8B).

Rabbit antisera prepared to CNBr III formed precipitin lines of complete identity between CNBr III, apoLP-Gln-II, and performic acid-oxidized apoLP-Gln-II (Fig. 8C). No differences were noted after reconstitution of CNBr III with phosphatidylethanolamine (Fig. 8D). Antisera to CNBr III did not form detectable immunoprecipitin lines with (CNBr IV)² in the presence or absence of phosphatidylethanolamine (Fig. 8, C and D).

DISCUSSION

We have undertaken a study of the determinants of lipid binding by the plasma lipoproteins. Since the binding of phospholipid to protein is probably fundamental to the association and transport of neutral lipids by the plasma lipoproteins, we have chosen to investigate the interaction between phosphatidylethanolamine and the apoproteins of HDL.

In this report, we have used two methods of examining the binding of phosphatidylethanolamine, namely, the apodehydrogenase assay and reconstitution with density gradient ultracentrifugation. We have also measured CD changes, since these have shown an interesting parallel with measurements of lipid-binding.

All of the apolipoproteins or peptide fragments thus examined which bind phospholipid exhibit an apparent increase in α-helical conformation in association with the lipid-binding. Fragments which do not bind phosphatidylcholine do not exhibit the increased negative ellipticity at 222 nm in the presence of lipid. It is not known whether this transformation in secondary structure is essential for the binding of lipid or whether it represents a secondary change as a consequence of a more hydrophobic environment. Nonetheless, the observed phenomenon may be a useful tool for the assessment of lipid-protein interactions.

Our definitions of lipid-binding, as described in this report, should be considered operational ones, since we are at present unable to relate the measurements to lipid-binding in intact HDL. The identification of an apparent lipid-binding site in a peptide fragment of the molecule, similarly, does not constitute definite proof of the function of the same site in the native lipoprotein. Nonetheless, the fact that one fragment of the molecule binds phospholipid while the other does not bind appreciably is of interest and suggests the occurrence of lipid-binding sites within the molecule. Further studies are needed to establish if there are unique lipid-binding sites within apolipoproteins and, if so, what are the minimal amino acid sequences required. It is interesting to note that while CNBr III inhibited reactivation of the apodehydrogenase in the presence of phosphatidylethanolamine, the fragment was less effective than apoLP-Gln-II. The findings suggest that long-range forces within the molecule may influence both phospholipid-binding and the folding of the molecule.

There was no evidence of significant binding of phosphatidylethanolamine by the NH₂-terminal fragment. Since reduction of the disulfide linkage of apoLP-Gln-II is known to decrease the α-helical content as judged by CD changes (2), it can be argued that the reoxidation of CNBr IV may alter the conformation...
so as to affect lipid-binding. The present study permitted us to examine the lipid-binding properties of the NH₂-terminal fragment with the disulfide bond intact, which had not previously been done. Oxidation of CNBr IV did not generate the ability to bind phospholipid. We cannot rule out the possibility that the conformation of (CNBr IV)₂ is different than that in the native lipoprotein. Chemical modification of the NH₂-terminal portion of apoLP-Glu-II has thus far failed to decrease the binding of phospholipid. Reduction and aminomethylation of the disulfide at residue 6 caused no change in the ability to inhibit the dehydrogenase, corroborating other experimental approaches from this laboratory (28). Similarly, performic acid oxidation, which both converts the methionine at position 26 to a sulfone and oxidizes the cystine to cysteic acid did not decrease binding of phospholipid and did not change the qualitative immunoprecipitin reactions. While both reduction with alkylation and performic acid oxidation reduced the apparent α helical content of the protein, the changes in secondary structure were largely overcome in the presence of phosphatidylcholine, despite the fact that the chemical alterations were not of a reversible nature. Whether this transformation is a secondary effect or of primary importance to phospholipid binding is of considerable interest and is under further study.

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