Mechanism of Lipid–Protein Interaction in the Plasma Lipoproteins: Relationship of Lipid-Binding Sites to Antigenic Sites in Apolipoprotein A-II†

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ABSTRACT: Previous studies have indicated that a major antigenic site of human plasma high-density apolipoprotein A-II (apoA-II) is contained in the COOH-terminal portion (residues 27–77) of the protein. Furthermore, all of the immunoreactivity in high-density lipoproteins (HDL) was detected by a specific radioimmunoassay. The present study is an attempt to define more precisely the antigenic structure of apoA-II. Tryptic fragments and synthetic peptides of apoA-II were prepared corresponding to specific regions of the protein. Immunoreactivity was assessed by competitive inhibition of the binding of [^{125}I]apoA-II to anti-apoA-II antisera. In the assays, tryptic fragments corresponding to residues 4–23 (the disulfide at Cys-6 was retained), residues 31–39, and residues 56–77 gave 28, 10, and 25% inhibition, respectively; a synthetic fragment representing residues 40–46 gave 10% inhibition. Further delineation of the antigenic reactive region(s) of the 56–77 fragment was achieved by chemical modification. After coupling glycine ethyl ester to Glu-59 and Glu-69 of the 56–77 tryptic fragment, the modified peptide did not form an immunoprecipitant line with anti-apoA-II. Since a synthetic peptide representing residues 60–77 gave an immunoprecipitate of complete identity to the 56–77 tryptic or synthetic fragments, we conclude that a major antigenic determinant is present between residues 60 and 77 and that Glu-69 is part of the determinant. Since previous studies have shown that the peptides corresponding to residues 1–26 and 56–77 do not bind phospholipid, the findings in this paper demonstrate the independence of such binding and the immunological reactions of apoA-II.

Apolipoprotein A-II (apoA-II)[1] is a major apoprotein of human plasma high-density lipoproteins (HDL). The apoprotein has two identical chains which are linked by a disulfide bond at position 6 (Figure 1). A previous study using a specific radioimmunoassay for apoA-II (Mao et al., 1975) indicated that the COOH-terminal (residues 27–77) region of apoA-II is immunologically more reactive than the NH_2-terminal (residues 1–26) region. With this assay procedure, the COOH-terminal fragment contained 70% and the NH_2-terminal 30% of the total immunoreactivity of apoA-II.

The aim of the present experiments was to define more precisely the antigenic sites of apoA-II and to determine their relationship to the lipid-binding sites of the apoprotein. Tryptic peptides and synthetic fragments of apoA-II were prepared and tested. One of the most important findings of this communication is that residues 56–77, which do not bind phospholipid (Mao et al., 1977), contain a major antigenic determinant. This result is discussed in relation to the previous finding (Mao et al., 1975; Schonfeld et al., 1977) that there is no masking of the immunoreactivity of apoA-II by the lipids in HDL.

Materials and Methods

Preparation of ApoA-II and Cyanogen Bromide Fragments. ApoA-II was isolated from apoHDL as described previously (Mao et al., 1975). Homogeneity of the apoA-II was determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Weber & Osborn, 1969) and urea (Davies, 1964), by amino acid analysis, and by double-immunodiffusion techniques using specific antisera prepared against each human apolipoprotein. The two cyanogen bromide fragments of apoA-II corresponding to residues 1–26 and 27–77 were obtained from reduced-carboxymethylated apoA-II (Jackson et al., 1973a,b).

Preparation of Antisera. Rabbit antisera were prepared against apoA-II by previously described methods (Lux et al., 1972b). Four rabbit anti-apoA-II antisera were pooled and partially purified by fractionation with 50% saturated ammonium sulfate. Double-immunodiffusion techniques were performed on immunodiffusion plates.

Preparation of Tryptic Peptides. The peptide corresponding to residues 4–23 was obtained from a tryptic digestion of apoA-II. ApoA-II (5 mg) in 1.0 mL of 0.05 M Tris-HCl, pH 8.0, was incubated with 0.1 mg of trypsin (TPCK, Worthington) for 2 h at 25 °C. The mixture was then chromatographed on a column (1 × 50 cm) of Sephadex G-50 equilibrated with 0.1 M ammonium bicarbonate, pH 8.0. The fractions (Figure 2) corresponding to the tryptic peptide (residues 4–23) were pooled and lyophilized.

The other tryptic peptides used in this study were prepared from the COOH-terminal cyanogen bromide fragment (residues 27–77). Fifty milligrams of 27–77 fragment was treated with 1 mg of trypsin for 30 min at 25 °C. The peptide mixture was fractionated by chromatography on a column (2.6 × 200 cm) of Sephadex G-50 in 0.1 M Tris-HCl, pH 8.0, containing 5.4 M urea. Zone A (Figure 3), which contained the tryptic peptide corresponding to residues 56–77, was further fractionated on DEAE-cellulose as described previously

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[1] Abbreviations used: apoA-II, apoprotein constituent of high-density lipoproteins (HDL); RIA, radioimmunoassay; GEE, glycine ethyl ester; DMPC, dimyristoylphosphatidylcholine; Ab, antibody.

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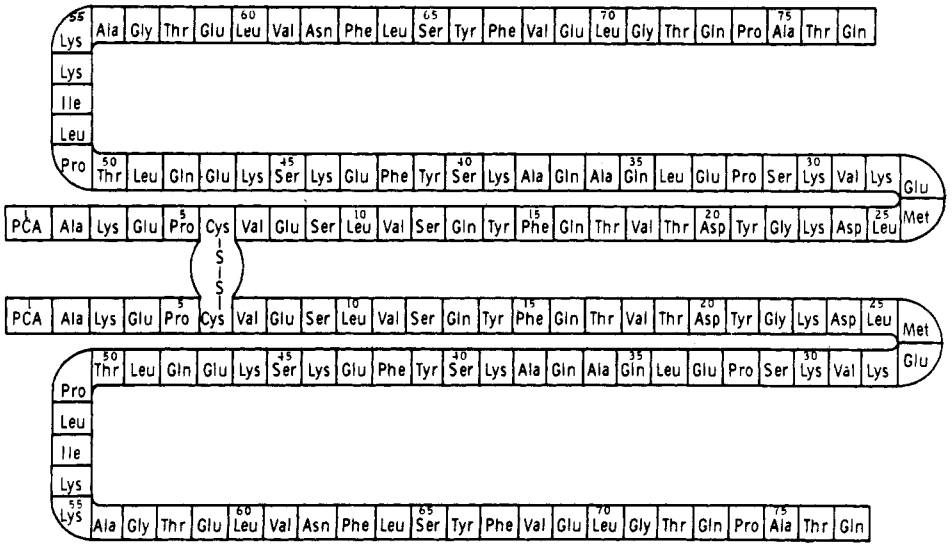

FIGURE 1: Amino acid sequence of apoA-II (as determined by Brewer et al., 1972; Lux et al., 1972a). The chains are connected by a disulfide bond at residue 6. PCA is pyrrolidonecarboxylic acid.

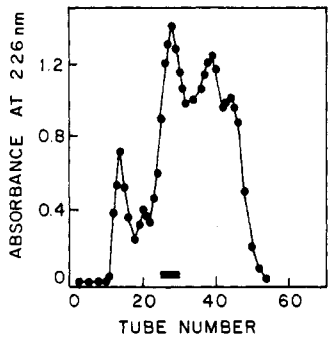

FIGURE 2: Chromatography of tryptic peptides of apoA-II on Sephadex G-50. ApoA-II (5 mg) in 1.0 mL of 0.05 M Tris-HCl, pH 8.0, was incubated with 0.1 mg of trypsin (TPCK, Worthington) for 2 h at 25 °C. The digest was applied directly to a column (1.0 × 50 cm) equilibrated with 0.1 M ammonium bicarbonate, pH 8.0. The column was operated at 25 mL/h; 1-mL fractions were collected. The fractions indicated by the bar were pooled and lyophilized.

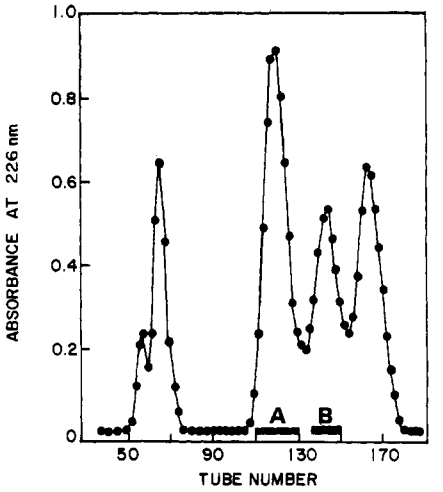

FIGURE 3: Chromatography on Sephadex G-50 of tryptic peptides of the cyanogen bromide peptide (residues 27–77) of apoA-II. The CNBr fragment (50 mg) was digested with 1 mg of trypsin (TPCK, Worthington) for 30 min at 23 °C. The digest was applied directly to a column (2.6 × 200 cm) equilibrated with 0.1 M Tris-HCl, pH 8.0, containing 5.4 M urea. The flow rate was 25 mL/h, and 5.6-mL fractions were collected. The fractions indicated by the bars were pooled, desalted on Bio-Gel P-2 in 0.1 M ammonium bicarbonate, and lyophilized.

(Mao et al., 1977). The remaining tryptic peptides (zone B) were separated on a column (1.6 × 30 cm) of DEAE-cellulose, equilibrated with 0.001 M ammonium bicarbonate, pH 8.0, by a procedure similar to that described by Brewer et al. (1972). The purity and concentration of the isolated peptides were determined by amino acid analysis.

Radioimmunoassay Procedure. Assays were performed in 12 × 75 mm disposable Falcon polypropylene tubes (Becton, Dickinson, and Co., Parsippany, NJ). All dilutions of peptides were made in a standard buffer containing 0.05 M potassium phosphate, 0.1 M NaCl, 0.01% merthiolate, 0.01% EDTA, and 5% nonimmune rabbit γ-globulin (pH 7.2). The assay, which is based on the inhibitory activity of the peptide to [^{125}I]-apoA-II bound to anti-apoA-II, has been described in detail (Mao et al., 1975). Human apoC-I was used as a control and did not show any inhibitory reactivity. In this assay, the percent inhibition = 100% − [(cpm of [^{125}I]apoA-II bound to Ab in the presence of fragment − background)/(cpm of [^{125}I]apoA-II bound to Ab − background)]. ApoA-II was iodinated with Na^{125}I by using a modification of the iodine monochloride method of McFarlane (1964). Iodine monochloride was prepared by diluting a stock solution with 2 M NaCl so as to provide one atom of iodine per molecule of apoA-II (Mao et al., 1975). The iodination reaction was allowed to proceed for 30 s at 4 °C. Chromatography of the labeled apoA-II on Sephadex G-150 yielded a single peak of radioactivity which coincided with the elution profile of unlabeled apoA-II. By double immunodiffusion, both [^{125}I]-apoA-II and apoA-II gave single precipitin lines of complete identity when tested against rabbit anti-apoA-II.

Preparation and Purification of Synthetic Fragments of ApoA-II. The peptides corresponding to residues 60–77 and 40–46 were synthesized by a modified solid-phase technique (Sparrow, 1976) on a Schwarz Bio-Research peptide synthesizer. The program used to incorporate the *tert*-butoxycarbonyl (Boc)-protected amino acid stepwise has been described (Mao et al., 1977). The peptides were simultaneously cleaved from the resin and deprotected with hydrogen fluoride (Mao et al., 1977). Truncated peptides were removed by chromatography on Bio-Gel P-10 equilibrated with 0.1 M Tris-HCl and 6.0 M urea, pH 8.2. The appropriate fractions were pooled and desalted on a column of Bio-Gel P-2 in 0.1 M ammonium bicarbonate, pH 8.0. Peptide 60–77 was further purified by ion-exchange chromatography on DEAE-cellulose as described in the legend to Figure 9. The purity of peptide

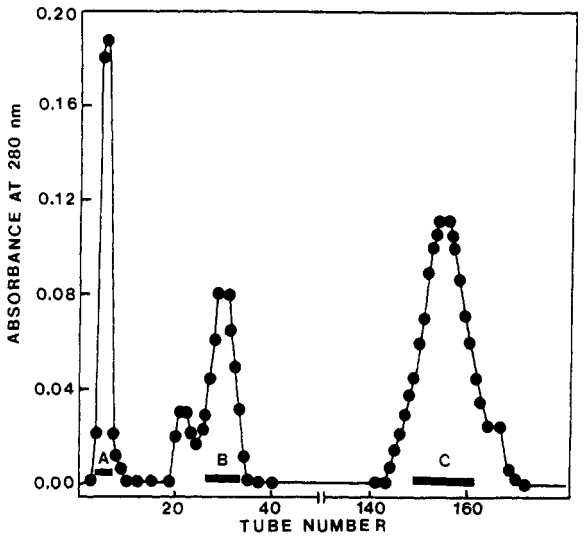

FIGURE 4: Chromatography of Sephadex G-50 zone B (Figure 3) on DEAE-cellulose. The sample was applied to a column (1.6 × 30 cm) of DEAE-cellulose (DE-52, Whatman) equilibrated with 0.001 M ammonium bicarbonate, pH 8.0, and was first eluted with a linear gradient consisting of 500 mL of the equilibration buffer and 500 mL of 0.01 M ammonium bicarbonate, pH 8.0. At tube 100 the column was eluted with a second gradient consisting of 500 mL of 0.01 M ammonium bicarbonate and 500 mL of 0.1 M ammonium bicarbonate, pH 8.0. The fractions represented in peak A correspond to residues 45–54 in apoA-II, peak B to residues 47–54, and peak C to residues 31–39.

60–77 was determined by polyacrylamide gel electrophoresis at pH 8.2, containing 8 M urea, and by amino acid analysis. The purity of the 40–46 fragment was examined by high-voltage paper electrophoresis in a pyridine–acetate buffer (pyridine–acetic acid–water, 1:10:289 v/v), pH 3.25.

Chemical Modification of Peptides 56–77 and 60–77. The peptides (0.5 μmol) were dissolved in 1.5 mL of 0.1 M NaCl solution, pH 4.7, containing 3 M guanidine hydrochloride; a 500 molar excess of glycine ethyl ester was added. To the mixture was then added a 50-fold molar excess of water-soluble carbodiimide [1,1-(dimethylamino)propyl-3-ethylcarbodiimide hydrochloride]. The reaction was allowed to progress for 2 h at 23 °C with the pH maintained at 4.7 by the addition of 0.1 M HCl. The mixture was applied to a column (1.0 × 100 cm) of Sephadex G-10 and eluted with 0.1 M ammonium bicarbonate, pH 8.0; the peptide-containing fractions were lyophilized. The extent of esterification was determined by amino acid analysis.

Results

Isolation of Tryptic Peptides. The chromatographic separation on Sephadex G-50 of the tryptic peptides of apoA-II is shown in Figure 2. The fractions indicated by the solid bar represented a pure peptide and corresponded to residues 4–23 in apoA-II as determined by amino acid analysis. The amino acid composition of the peptide is given in Table I and was consistent with the theoretical values.

The COOH-terminal cyanogen bromide fragment (residues 27–77) was digested with trypsin, and the mixture was chromatographed on Sephadex G-50 in 0.1 M Tris-HCl, pH 8.0, containing 5.4 M urea (Figure 3). The fractions indicated as zone A had an amino acid composition consistent with the sequence of residues 56–77, but in addition it also contained 0.4 residue of lysine, suggesting that trypsin had partially cleaved between residues 54 and 55. Peptide fragments 56–77 and 55–77 were separated by DEAE-cellulose chromatography (Mao et al., 1977). Zone B (Figure 3) contained several

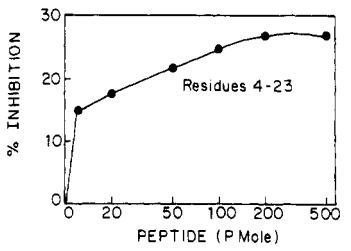

FIGURE 5: Immunoreactivity of peptide 4–23 as determined by quantitative inhibition assay. The assay contained 2 ng of [^{125}I]-apoA-II. The anti-apoA-II was diluted 1:1000; the procedure is as described previously (Mao et al., 1975) and under Materials and Methods.

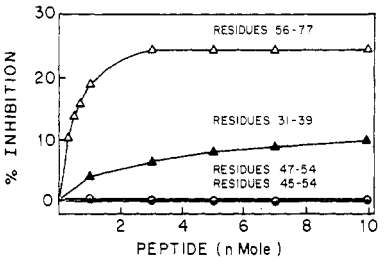

FIGURE 6: Immunoreactivity of tryptic peptides obtained from the CNBr fragment (residues 27–77). The inhibition assay contained 10 ng of [^{125}I]apoA-II. The anti-apoA-II antisera were diluted 1:500.

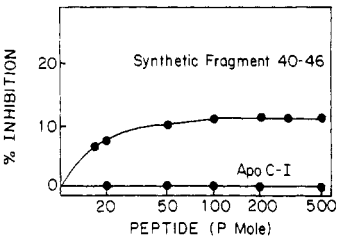

FIGURE 7: Immunoreactivity of synthetic peptide 40–46. The apoC-I used as the control was isolated from human VLDL. The inhibition assay contained 2 ng of [^{125}I]apoA-II. The anti-apoA-II antisera were diluted 1:1000.

peptides which were further purified on DEAE-cellulose (Figure 4); three major fractions were detected. As determined by amino acid analysis, fraction A corresponded to the peptide representing residues 45–54, fraction B to residues 47–54, and fraction C to residues 31–39. The amino acid composition of each peptide was consistent with its corresponding sequence in apoA-II (Table I).

Immunoreactivity of the Tryptic Fragments of ApoA-II. We previously found (Mao et al., 1975) that CNBr fragment 1–26 has approximately 30% and fragment 27–77 70% of the activity of apoA-II in inhibiting antibody binding to [^{125}I]-apoA-II. The tryptic fragments of apoA-II isolated as described above were compared for their immunoreactivity in the inhibitory assay (Figure 5). Peptide 4–23 inhibited [^{125}I]apoA-II binding to anti-apoA-II by 27% when 200 pmol of peptide was added. This inhibition was virtually indistinguishable from the results with fragment 1–26 (Mao et al., 1975). The results with the remaining tryptic fragments are shown in Figure 6. At 3 nmol fragment 56–77 gave 25% inhibition, at 10 nmol peptide 31–39 gave only 10% and the remaining peptides 45–54 and 47–54 were inactive.

Synthesis, Purification, and Immunoreactivity of Synthetic Peptide 40–46. Since peptides 45–54 and 47–54 were inactive in the inhibitory assay while peptide 31–39 had 10% activity, we synthesized the peptide corresponding to residues 40–46 for purposes of comparison. The amino acid composition of the isolated peptide is given in Table I; the peptide gave a single

Table I: Amino Acid Compositions of Synthetic and Native Fragments of ApoA-II[a]

amino acid	native fragment 4–23	native fragment 31–39	native fragment 45–54	native fragment 47–54	native fragment 56–77	synthetic fragment 40–46	synthetic fragment 60–77
Asp	1.0 (1)				1.1 (1)		0.9 (1)
Thr	1.8 (2)		1.0 (1)	1.1 (1)	2.9 (3)		1.8 (2)
Ser	1.8 (2)	1.0 (1)	0.9 (1)		0.9 (1)	1.8 (2)	0.9 (1)
Glu	4.2 (4)	3.1 (3)	2.0 (2)	1.9 (2)	4.3 (4)	1.1 (1)	3.4 (3)
Pro	1.2 (1)	0.9 (1)	1.0 (1)	0.9 (1)	1.1 (1)		1.0 (1)
Gly	1.1 (1)				2.1 (2)		1.1 (1)
Ala		2.1 (2)			2.0 (2)		1.0 (1)
$^1/_2$-Cys	0.3 (1)						
Val	2.8 (3)				1.6 (2)		1.8 (2)
Ile			1.0 (1)	1.0 (1)			
Leu	1.2 (1)	1.2 (1)	2.2 (2)	2.1 (2)	2.9 (3)		2.8 (3)
Tyr	2.0 (2)				1.1 (1)	1.0 (1)	1.0 (1)
Phe	0.9 (1)				2.0 (2)	1.0 (1)	2.0 (2)
Lys	0.9 (1)	1.0 (1)	1.9 (2)	1.1 (1)		2.0 (2)	
total	20	9	10	8	22	7	18

[a] Amino acid analyses were obtained on peptide samples subjected to hydrolysis (24 h, 110 °C) in sealed evacuated tubes employing 6 N HCl. Analysis was performed on a Beckman Model 117 or 119 analyzer equipped with an Autolab integrator. The values represent the average of two determinations; the values in parentheses are the theoretical values.

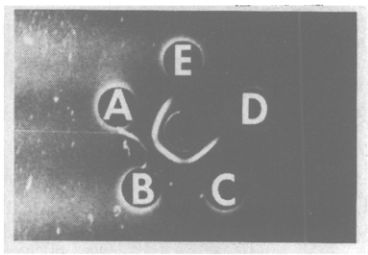

FIGURE 8: Double-immunodiffusion analysis of peptides 56–77, 60–77, and derivatives. The wells contained 10 μg in 10 μL of (A) synthetic peptide 56–77, (B) tryptic peptide 56–77, (C) synthetic peptide 60–77, (D) GEE–56–77, and (E) GEE–60–77.

spot when subjected to high-voltage electrophoresis at pH 3.7. The immunoreactivity of peptide 40–46 is shown in Figure 7. The peptide gave 11% inhibition of the binding of [^{125}I]apoA-II to anti-apoA-II.

Effect of Chemical Modification on the Immunoreactivity of Peptide 56–77. For further delineation of the antigenic reactive region of peptide 56–77, chemical modification of selected amino acid residues was attempted. Peptide 56–77 was coupled to glycine ethyl ester (GEE) in the presence of the promoting reagent water-soluble carbodiimide. As determined by the glycine content after amino acid analysis, Glu-59 and Glu-69 were completely coupled. When the derivatives were tested against anti-apoA-II antibodies, no precipitin line formed in the double-immunodiffusion gel (Figure 8). This finding indicated that either one or both of the glutamic acid residues 59 and 69 were involved in antigen–antibody interaction. If Glu-59 were located in the antigenic reactive region of 56–77, then synthesis of a peptide corresponding to residues 60–77 would be expected to produce a fragment with less immunoreactivity. The peptide corresponding to residues 60–77 was prepared by the solid-phase method (see Materials and Methods). Chromatography of the synthetic fragment on DEAE-cellulose gave a major peptide fraction (Figure 9). Polyacrylamide gel electrophoresis at pH 8.2 in 8 M urea gave a single peptide staining band (inset, Figure 9). The amino acid composition of the isolated peptide (Table I) was consistent with the theoretical values. When the synthetic peptide 60–77 was tested with anti-apoA-II, a precipitin line of complete identity to fragment 56–77 was obtained (Figure 8). Chemical modification of Glu-69 by coupling with glycine ethyl ester completely

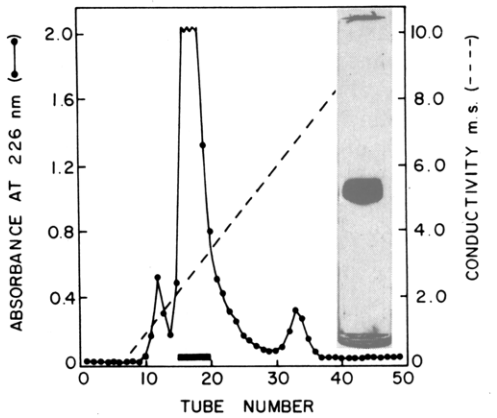

FIGURE 9: Purification of synthetic peptide 60–77 of apoA-II by DEAE-cellulose. The peptide was applied to a column (1.6 × 30 cm) of DEAE-cellulose (DE-52 from Whatman) at a concentration of 5 mg/mL. After the sample entered the resin, it was eluted with a gradient of 500 mL of 0.01 M Tris-HCl, pH 8.2, and 500 mL of the same buffer containing 0.2 M NaCl; 5-mL fractions were collected, and the flow rate was 25 mL/h. The fractions under the bar were pooled, desalted, and characterized by amino acid analysis and polyacrylamide gel electrophoresis in 8 M urea.

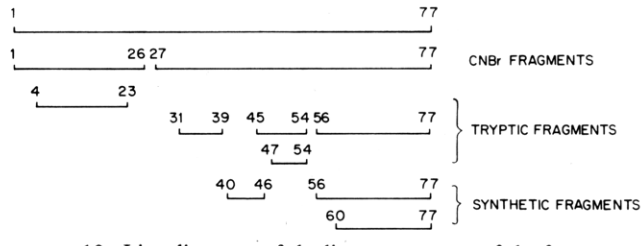

FIGURE 10: Line diagrams of the linear sequences of the fragments tested in these studies of the immunoreactivity of apoA-II.

abolished the immunochemical reactivity of the 60–77 fragment (Figure 8). This result suggests that Glu-69 rather than Glu-59 is involved in the antigenic reactive region of 56–77.

Discussion

Identification of Antigenic Regions in ApoA-II. The primary sequences of all peptides tested, both native and synthetic, are illustrated in Figure 10. We have previously shown (Mao et al., 1975) that the NH_2-terminal cyanogen

bromide fragment (residues 1–26) of apoA-II has a maximum immunoreactivity of ~30% as compared to apoA-II. We have now extended these studies and have found that the tryptic peptide corresponding to residues 4–23 with the disulfide intact has 27% immunoreactivity. Because the inhibitory activity of 4–23 is similar to residues 1–26 (Mao et al., 1975), we suggest that the antigenic reactive region of the NH_2-terminal portion of apoA-II is located between residues 4 and 23.

The COOH-terminal cyanogen bromide fragment (residues 27–77) contained ~70% of the immunoreactivity of apoA-II (Mao et al., 1975). Of the tryptic peptides which were tested in the present study, residues 31–39 gave 10% immunoreactivity, residues 56–77, 25%, and synthetic peptide 40–46, 11%. Tryptic peptides 45–54 and 47–54 were inactive. Whether residues 40–46 contain a separate antigenic determinant or represent a continuation of the 31–39 antigenic determinant is not known. In either case, the results indicate that there is a second antigenic reactive site between residues 31 and 46.

By RIA, the tryptic peptide corresponding to residues 56–77 gave a maximum of 25% inhibition, indicating that the COOH-terminal portion of the apoprotein contained an antigenic determinant. Chemical modification of the 56–77 fragment by coupling of Glu-59 and Glu-69 with glycine ethyl ester completely abolished the immunoreactivity of the fragment, suggesting that one or both of these glutamic acid residues are part of the antigenic site(s). The synthetic fragment 60–77 gave a precipitin line of complete identity (Figure 8) with the 56–77 peptide. Since glycine ethyl ester coupled to Glu 69 in the 60–77 peptide abolished the immunoreactivity, these findings indicate that Glu-69 is in the determinant and that Glu-59 is not. Therefore, we suggest that a third antigenic determinant is located between residues 60 and 77 in apoA-II. An important finding of these studies is that a major antigenic reactive region of apoA-II contains no positively charged group, i.e., no lysine and arginine, which are reported to occur in the five antigenic reactive regions of sperm whale myoglobin (Atassi, 1975).

Relationship between Antigenic Sites and Lipid-Binding Sites. In previous reports (Segrest et al., 1974; Jackson et al., 1975), it was suggested that apoA-II as well as the other plasma apolipoproteins contains amphipathic α-helical segments which represent the basic lipid-binding units for this class of lipid-associating proteins. On the basis of model building (Segrest et al., 1974), we predicted that residues 18–30 are amphipathic and should represent a lipid-binding site in apoA-II. Recently, Chen et al. (1979) have shown that a synthetic fragment containing residues 12–31 binds to dimyristoylphosphatidylcholine, whereas the NH_2-terminal cyanogen bromide fragment (residues 1–26) does not bind but is immunoreactive. Since there is an overlap in the sequence of the lipid-binding and antigenic peptides we cannot, at present, determine whether the two sites are contained in two different regions of the apoprotein. Although we predicted residues 39–47 to be amphipathic and to be involved in phospholipid binding, the relationship between the second antigenic region in residues 31–46 and lipid binding is unknown since the corresponding synthetic peptide has not been prepared.

In the present report we find that a third antigenic site is contained in residues 60–77. Previously, we have shown that a tryptic or synthetic peptide corresponding to residues 56–77 does not bind phospholipid (Mao et al., 1977). Therefore, we conclude that the lipid-binding site and the antigenic site are independent in this region of apoA-II. Our results also support the finding by Mao et al. (1975) and Schonfeld et al. (1977) that lipid does not affect the antigenic reactivity of apoA-II. Taken together, these results suggest that the conformational changes resulting from the interaction with lipid do not affect the secondary structure of the antigenic sites, and thus immunoreactivity is preserved.

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