Further Characterization of Apolipoproteins from the Human Plasma Very Low Density Lipoproteins

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SUMMARY

Valine apolipoprotein, glutamic acid apolipoprotein, and alanine apolipoprotein (apoLP-Ala) (designated by their COOH-terminal amino acids) are three small apolipoproteins which constitute more than half of the protein in human plasma very low density lipoprotein. These proteins were isolated free of lipid by techniques previously described. As determined by sedimentation equilibrium analysis and estimated from amino acid analyses, the approximate molecular weights of the apolipoproteins were: valine apolipoprotein, 7,000; glutamic acid apolipoprotein, 10,000; and apoLP-Ala, 10,000. Circular dichroism and optical rotatory dispersion spectra for valine apolipoprotein were consistent with a high content of a helix, while the spectra obtained with apoLP-Ala suggested its structure to be mainly of random coil. Previously reported polymorphism of apoLP-Ala was found to be attributed to differences in content of sialic acid.

The very low density lipoproteins of human plasma contain no less than five different apolipoproteins (1, 2). These include the apolipoproteins characteristic of low density and high density lipoproteins (3). Three other apolipoproteins constitute over half of the total protein component of VLD (very low density) lipoproteins (2). These three proteins are usually designated by their COOH-terminal amino acids as apoLP-Val, apoLP-Glu, and apoLP-Ala. The amino-terminal residue of both apoLP-Val and apoLP-Glu is threonine and of apoLP-Ala is serine. New data obtained in the further characterization of these three apolipoproteins are the subject of this report. Molecular weights for all three proteins have been determined by sedimentation equilibrium analysis in the ultracentrifuge, and these values have been compared to molecular weights estimated from amino acid analyses. Measurements of ultraviolet absorption, optical rotatory dispersion, and circular dichroism have been used to study the structure of apoLP-Val and apoLP-Ala. A basis for apparent polymorphism of apoLP-Ala has also been established. This protein is present in at least two forms that are immunochemically identical and have the same amino acid composition, NH₂ and COOH-terminal groups, and CD and ORD spectra. They are separable into two bands on polyacrylamide electrophoresis and two fractions by ion exchange chromatography. This chromatographic behavior has been shown to result from differences in carbohydrate composition.

EXPERIMENTAL PROCEDURE

Materials—Neuraminidase from Vibrio cholerae (500 units per ml) was supplied by Calbiochem, and N-acetylneuraminic acid by Pfanstiehl Laboratories, Inc., Waukegan, Illinois. Ultrapure guanidine was purchased from Mann, and dialysis casing (number 16) from Union Carbide. The sources of the remainder of the materials were those previously reported (1).

Source and Preparation of Apolipoproteins The VLD lipoproteins were obtained from subjects with types IV and V hyperlipoproteinemia (4). The techniques of isolation, removal of the lipid, and solubilization were identical with those previously described (1). The VLD apolipoproteins were separated chromatographically according to a revised scheme which permitted a better yield of all three apolipoproteins and a more satisfactory purification of apoLP-Glu (2). The criteria for purity of the apolipoproteins were the presence of a single band on polyacrylamide electrophoresis, a single precipitin line obtained with specific antisera by the Ouchterlony technique, and a characteristic amino acid composition after acid hydrolysis. The techniques of electrophoresis in polyacrylamide gels and immunochemical analysis were employed as previously described (1).

Optical Studies—Samples of apoLP-Ala and apoLP-Val were lyophilized once in 0.1 M NH₄HCO₃ and three times in distilled water, and stored over P₂O₅ under vacuum to constant weight. Absorbance was determined in 0.02 M potassium phosphate buffer (pH 7.5) in a Beckman DU spectrophotometer at a light path of 1 cm. Linear plots of absorbance against concentration were obtained for solutions containing 0.0025 to 1.0 mg per ml. The CD spectra were recorded at 23 to 27° with a Cary model 60 spectropolarimeter, equipped with a Pockels cell (5). The mean residue ellipticity [θ], in degrees of square centimeters per decopeptide, was calculated from the following equation:

\[ [\theta] = \frac{1000 \times \text{Absorbance} \times C \times L}{\text{Concentration} \times S} \]

where C is the concentration of the protein in mg/ml, L is the light path in cm, and S is the specific ellipticity in degrees per decopeptide per mg/ml.
FIG. 1. Sedimentation equilibrium experiments with apoLP-Val in guanidine-HCl solutions of 3 M (A) and 7 M (B). The two experiments with 7 M guanidine-HCl were performed at slightly different protein concentrations. Linear plots of ln absorbance (280 nm) against the square of the distance (r) from the axis of rotation were obtained in each experiment.

where $\theta$ is the observed ellipticity. The mean residue weight ($MRW$) was 111 g per mole for apoLP-Val and 115 g per mole for apoLP-Ala. The path length ($l$) was 0.5 mm in all experiments. The concentration of the protein ($c$) varied from $0.25 \times 10^{-3}$ to $1.0 \times 10^{-3}$ g per ml in 0.05 M potassium phosphate buffer (pH 7.5). ORD spectra were also recorded in the Cary model 60 with the equipment in the ORD mode (6). The conditions were otherwise identical with those used for CD measurements. The data were plotted as mean residue rotation [$\theta$] where:

$$\theta = \frac{\theta^0}{10} \times \frac{MRW}{lec}$$

and $\theta^0$, $l$, and $c$ are the same as those used in the relationship defining molar ellipticity ($\theta$). $\alpha$ is the observed rotation in degrees at wave length $\lambda$.

**Sedimentation Equilibrium Experiments**—All studies were performed in the Beckman-Spinco model E analytical ultracentrifuge equipped with electronic speed control, absorption optics, photoelectric scanning, and multiplex systems. A titanium rotor (type AN-G) containing six compartments was used. Five of the compartments contained double sector cells with sapphire windows, and the sixth held a counterbalance with calibrated reference windows 1.570 cm apart. Protein samples were lyophilized in 0.05 M NH$_4$HCO$_3$ and dissolved at concentrations of 1 mg per ml in 0.1 M NaCl, 0.02 M Tris-HCl at pH 8.6, and guanidine-HCl in concentrations of 3, 5, or 7 M (7). The samples were dialyzed in dialysis casing against 50 volumes of the buffer at 4°C, the dialysates being changed three times at intervals of 24 hours. The density of the final dialysate was determined by pycnometry at 24°C and corrected by standard tables to 20°C. Immediately prior to centrifugation, the protein solutions were diluted with the final dialysate to give absorbances of 0.35 to 0.45 at 280 nm. The solution sector contained 0.20 ml and the solvent sector 0.25 ml of the final dialysate. Equilibrium was judged to be present and the final scans taken when the plot of absorbance against distance from the axis of rotation did not change for at least 10 hours. The length of most experiments was 44 hours, the rotor speed was 28,000 rpm, and the temperature 20°C.

**Amino Acid Analyses**—Aliquots (0.5 mg) of the same protein preparations utilized for sedimentation equilibrium were hydrolyzed in constant boiling glass-distilled HCl at 110°C for 12, 24, 48, and 72 hours. Norleucine and homoserine were added as internal standards before the aliquots were taken for hydrolysis. Other aliquots of protein were subjected to performic acid and hydrolyzed as above for 24 hours. The amino acid content was determined spectrophotometrically by the method of Edelhoch (10).
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Sedimentation Equilibrium Experiments—When apoLP-Val, apoLP-Glu, or apoLP-Ala were centrifuged in the absence of guanidine, the slope of the curve relating absorbance to the square of the distance from the axis of rotation (42) increased as measurements were made from the meniscus to the bottom of the cell. This phenomenon was considered most likely due to intramolecular association since each of the protein preparations appeared as a single band on polyacrylamide gel electrophoresis (15% gel, 8 M urea). Accordingly, guanidine-HCl was added to the buffers to offset apparent aggregation. The technique used by Schachman and Edelstein (7) for analysis of interacting systems to obtain molecular weight was then applied. Linear plots of ln absorbance versus \( r^2 \) were obtained for apoLP-Glu and Ala in 3, 5, and 7 M guanidine-HCl (Figs. 1 to 3). Plots of ln absorbance versus \( r^2 \) were obtained on apoLP-Val only at 3 M and two different protein concentrations in 7 M guanidine. The quantity \( M_e(1 - \bar{V}_p) \) for each of the guanidine-HCl concentrations was calculated from the relation

\[
\ln A = 0.704 \sqrt{I/\rho c}
\]

FIG. 3. Sedimentation equilibrium experiments with apoLP-Ala with conditions identical with those of experiments presented in Fig. 2.

Sialic Acid Determinations—Sialic acid was released by incubation of the protein in 0.1 M H\(_2\)SO\(_4\) for 1 hour at 80° (11) or by hydrolysis with neuraminidase (\( V.\) cholerae) at 25° in 0.08 M ammonium acetate at pH 5.0 (12). It was also necessary to add 0.002 M sodium decyl sulfate to maintain the solubility of apoLP-Ala at this pH. Enzyme (10 units) was added for each mg of glycoprotein substrate. Free sialic acid was determined by the thiobarbituric acid method with N-acetylneuraminic acid as standard (13).

RESULTS

Sedimentation Equilibrium Experiments—When apoLP-Val, apoLP-Glu, or apoLP-Ala were centrifuged in the absence of guanidine, the slope of the curve relating absorbance to the square of the distance from the axis of rotation (42) increased as measurements were made from the meniscus to the bottom of the cell. This phenomenon was considered most likely due to intramolecular association since each of the protein preparations appeared as a single band on polyacrylamide gel electrophoresis (15% gel, 8 M urea). Accordingly, guanidine-HCl was added to the buffers to offset apparent aggregation. The technique used by Schachman and Edelstein (7) for analysis of interacting systems to obtain molecular weight was then applied. Linear plots of ln absorbance versus \( r^2 \) were obtained for apoLP-Glu and Ala in 3, 5, and 7 M guanidine-HCl (Figs. 1 to 3). Plots of ln absorbance versus \( r^2 \) were obtained on apoLP-Val only at 3 M and two different protein concentrations in 7 M guanidine. The quantity \( M_e(1 - \bar{V}_p) \) for each of the guanidine-HCl concentrations was calculated from the relation

\[
\ln A = 0.704 \sqrt{I/\rho c}
\]

Fig. 4. Plot of \( M_e(1 - \bar{V}_p) \) against density for apoLP-Glu. Slopes of the plots shown in Fig. 2 for the apoLP-Glu protein were used to obtain values for \( M_e(1 - \bar{V}_p) \) for each solution of guanidine-HCl. These values were then plotted against the density of the respective guanidine-HCl solutions. \( \bar{V}_c \) is obtained from the intercept on the abscissa, which gives a value of \( \rho_c \) and the relation \( 1/\rho_c = \bar{V}_c \). Extrapolation to the intercept on the ordinate gives a value for \( M_e(1 - \bar{V}_p) \) in the theoretical solution with density one and thus allows calculation of a value for \( M_p \) for apoLP-Glu and its preferentially bound small molecular weight substances.

\[
M_e(1 - \bar{V}_p) = \frac{2RT}{c^2} \left( \frac{d \ln \text{absissia}}{d \rho^2} \right)
\]

A straight line relation was obtained when \( M_e(1 - \bar{V}_p) \) was plotted against the density of the respective solution (Figs. 4 and 5) for apoLP-Glu and -Ala. The partial specific volume of the complexes (\( \bar{V}_c \)) was calculated from the intercept of these plots on the abscissa for each of the proteins studied and is given in Table I. Since only two points were obtained for apoLP-Val, \( \bar{V}_e \) was not calculated for this protein. The partial specific volumes of the proteins calculated by the method of Cohn and Edsall (14) from the amino acid composition are also given in Table I as \( \bar{V}_p \). If it is assumed that the difference between \( \bar{V}_c \) and \( \bar{V}_p \) is due entirely to preferentially bound water, the portion of the weight of the complex due to bound water (\( x \)) can be used to obtain a molecular weight for the protein (\( M_p \)) by the relation

\[
M_p = M_e / (1 - \bar{V}_p)
\]

The values of \( M_e \) and \( M_p \) obtained in a series of experiments are
M(I-v,p) vs $\rho$

Values of $M_\rho$ were calculated by Equation A below from the slopes of the plots of ln absorbance against $r^2$ obtained in the guanidine-HCl solutions of density ($\rho$) indicated. $M_\rho$ was calculated using Equation B and the values for bound water from Table I.

Equation A  
$$M_\rho = \frac{2RT}{(1 - V_\rho)c_1}$$

Equation B  
$$M_\rho = \frac{2RT}{(1 + x)}$$

$\rho$ = bound H$_2$O in g:g protein

| Experiment no. | $\rho$ | $M_\rho$ | $M_\rho$ |
|---------------|-------|---------|---------|
| ApoLP-Glu     |       |         |         |
| 1             | 1.0879 | 12,640  | 9,930   |
| 2             | 1.1309 | 12,680  | 9,970   |
| 3             | 1.1666 | 12,440  | 9,770   |
| ApoLP-Ala     |       |         |         |
| 1             | 1.0879 | 14,540  | 10,060  |
| 2             | 1.1309 | 14,090  | 9,740   |
| 3             | 1.1666 | 14,370  | 9,940   |

Table I  

The values of $V_\rho$ are the partial specific volumes calculated from the amino acid content. The partial specific volumes from the sedimentation equilibrium experiments ($\bar{V}_\rho$) by the method of Schachman and Edelstein (7) are considerably higher than the corresponding values for $V_\rho$. If this difference is assumed to be due entirely to bound water, the quantities of water present may be calculated and are shown below. An approximate reduction in molecular weight is obtained by subtracting the bound water from the molecular weight of the complex ($M_c$) to obtain $M_\rho$. The molecular weight obtained from the amino acid analyses of apoLP-Val and apoLP-Ala ($M_{AA}$) compare favorably to the corresponding values for $M_\rho$. A value of $M_{AA}$ for apoLP-Glu was not calculated in the absence of tryptophan measurements.

|         | Valine very low density apolipoprotein | Glutamic acid very low density apolipoprotein | Alanine very low density apolipoprotein |
|---------|--------------------------------------|---------------------------------------------|---------------------------------------|
| $V_\rho$| 0.741                                | 0.725                                       | 0.726                                 |
| $\bar{V}_\rho$ | 0.784                              | 0.792                                       |                                       |
| Bound water (g:g) | 0.27                               | 0.45                                        |                                       |
| $M_c$  | 9,930                                | 9,960                                       |                                       |
| $M_\rho$ | 12,640                              | 14,400                                      |                                       |
| Number of amino acids | 62                                 | 81                                          |                                       |
| $M_{AA}$| 7,200                                | 9,100                                       |                                       |

Amino Acid Composition—The composition of each of the polypeptides differed in almost every amino acid (Table III).

Fig. 6. Neuraminidase digestion of apoLP-Ala. Equal weight of the $D_3$ and $D_4$ forms of apoLP-Ala were mixed and incubated with neuraminidase as described in the text. At the times indicated, aliquots were taken for analysis of released sialic acid and for polyacrylamide gel electrophoresis. The height of the open bars represents the theoretical content of sialic acid present in the mixture, assuming 2 moles per mole of protein as $D_3$ and 1 mole per mole as $D_4$ (46 mg of sialic acid per g of protein). The solid portion of the bar represents the measured release of sialic acid at the time given. A third, more slowly migrating band on electrophoresis appeared in the first aliquot after 30 min of incubation, and after 24 hours, when 90% of the theoretical total had been released, only this band was prominent.

Fig. 5. Plot of $M_\rho$ against density for apoLP-Ala. Plot of $M_c$ against the density of the guanidine-HCl solutions used as calculated from the slopes of the plots in Fig. 3 for apoLP-Ala. See legend for Fig. 4.

Fig. 3. Effect of neuraminidase on $D_3 + D_4$.

Values of $V_\rho$ obtained by the use of the remaining amino acids alone, since the $V$ of tryptophan itself (0.74) is so close to this value.
Amino acid content for each of the apolipoproteins. The values are given in moles per mole of protein, and each represents the average of values obtained from analyses of 12-, 24-, 48-, and 72-hour hydrolysates (HCl, 10°) when no trend in the values suggested destruction or delayed release. When the content diminished with time as for serine and tyrosine, the value given was obtained by extrapolation to zero time. Tryptophan was determined spectrometrically. "Cysteine + cystine" values were shown not to be present by the absence of cysteic acid in hydrolysates of performic acid-oxidized protein. All analyses were performed on samples from the same individual. The internal standards were norleucine and homogarginine. Moles per mole of protein were calculated as described under "Results."

|                | ApoLP-Val | Calculated | ApoLP-Glu | Calculated | ApoLP-Ala | Calculated |
|----------------|-----------|------------|-----------|------------|-----------|------------|
|                | mole/mole| mole/mole | mole/mole| mole/mole | mole/mole| mole/mole  |
| Asp            | 2.17      | 2.07      | 2.10      | 2.00      | 2.05      | 2.06      |
| Thr            | 1.30      | 1.20      | 1.18      | 1.19      | 1.05      | 1.06      |
| Ser            | 3.07      | 2.93      | 2.79      | 2.46      | 3.37      | 3.31      |
| Glu            | 4.50      | 4.32      | 4.44      | 4.23      | 3.37      | 3.31      |
| Pro            | 1.04      | 0.83      | 0.90      | 0.82      | 0.46      | 0.47      |
| Gly            | 0.46      | 0.44      | 0.47      | 0.44      | 0.47      | 0.48      |
| Ala            | 1.46      | 1.39      | 1.46      | 1.35      | 3.92      | 3.88      |
| Val            | 0.74      | 0.85      | 0.88      | 0.84      | 3.92      | 3.88      |
| Met            | 0.43      | 0.41      | 0.42      | 0.40      | 0.47      | 0.48      |
| Thr            | 1.17      | 1.32      | 1.38      | 1.39      | 0.70      | 0.71      |
| Leu            | 2.86      | 2.86      | 2.95      | 2.98      | 1.75      | 1.76      |
| Tyr            | 0         | 0         | 0         | 0         | 0.56      | 0.56      |
| Phe            | 1.33      | 1.34      | 1.32      | 1.30      | 3.07      | 3.07      |
| Lys            | 4.00      | 4.10      | 4.34      | 4.35      | 1.75      | 1.77      |
| His            | 0         | 0         | 0         | 0         | 1.22      | 1.22      |
| Arg            | 1.20      | 1.36      | 1.25      | 1.28      | 0.29      | 0.30      |
| Cysteine + cystine | 1.16 | 0.22      | 0.22      | 0.22      | 0.69      | 0.72      |

* Values obtained by extrapolation to zero time.
* Spectrometrical determination by the method of Edelhoch (40).
* Analysis for cysteic acid after 24-hour acid hydrolysis of performic acid-treated protein.
protein is due to the presence of the 3 moles of tryptophan and 2 moles of tyrosine per mole of protein. When analyzed in 6 M guanidine, no shift was noted in the position or magnitude of the maximum at 280 nm and shoulder at 290 nm obtained with either protein in the absence of guanidine. This suggests that the exposure of the aromatic group of tryptophan and tyrosine to the solvent is not changed by exposure to guanidine.

The circular dichroic spectrum of apoLP-Val included strong and well resolved bands at 222 nm and 207 nm (Fig. 8). The band at 222 nm is frequently associated with the $\pi \rightarrow \pi$ transition of the peptide bond in the $\alpha$ helix. If the intensity of absorption at this wavelength was produced by this configuration of the peptide bond, these studies suggested a significant content of $\alpha$ helical structure. The ORD spectrum of apoLP-Val was also consistent with some content of $\alpha$ helix, there being a minimum at 232 nm and a shoulder at 212 to 216 nm. The CD and ORD spectra of apoLP-Ala were quite different from those of apoLP-Val (Fig. 9). The major CD band was at 204 nm with a shoulder at 220 to 230 nm. ORD measurements on the same sample revealed a negative Cotton effect with a minimum at 228 to 230 nm. While these spectra suggest that apoLP-Ala contains mainly disordered structure, they do not exclude small contributions from helical or $\beta$ structure.

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Fig. 7. Absorption spectra of apoLP-Ala (A) (0.49 mg per ml) and of apoLP-Val (B) (0.51 mg per ml), determined in 0.02 M potassium phosphate, pH 7.5, at 20°.

Fig. 8. The CD spectra of apoLP-Ala (---) and apoLP-Val (---) in 0.02 M potassium phosphate buffer, pH 7.5.

Fig. 9. ORD spectra of apoLP-Ala (---) and of apoLP-Val (---) in 0.02 M potassium phosphate buffer, pH 7.5.
DISCUSSION

The molecular weights determined for the three VLD apolipoproteins, apoLP-Val, apoLP-Glu, and apoLP-Ala were approximately 7,000, 10,000, and 10,000, respectively. A previous estimate of 14,000 for apoLP-Ala had been based solely on the yield of terminal residues by carboxypeptidase (1). These are the smallest of the apolipoproteins thus far isolated from human plasma. The two major high density apolipoproteins, threonine apolipoprotein and glutamine apolipoprotein, have molecular weights of approximately 15,000 (15). All of the apolipoproteins have a strong tendency to aggregate, and measurements of molecular weights by sedimentation equilibrium have required the presence of dissociating substances such as guanidine or urea (15). This requires application of a technique such as that of Schachman and Edelstein (7) in the determination of molecular weights of approximately 15,000 (15). The presence of terminal residues by carboxypeptidase (1) and estimate of 14,000 for apoLP-Ala had been based solely on the obtained in these experiments are high compared to some other proteins, and it is therefore reassuring that the estimates of molecular weight obtained from amino acid composition agree well with those obtained by ultracentrifugation.

It is apparent that the VLD lipoprotein complexes must contain a large number of different protein molecules. For example, the VLD particle of molecular weight 15 x 10^6 contains about 75 to 100 molecules of the smaller VLD apolipoproteins (17). They obtained a total of six different fractions by DEAE-cellulose chromatography. From the amino acid composition alone, none of these can be positively identified as corresponding to the proteins referred to here as apoLP-Val or apoLP-Glu, but the composition of Peaks 1 and 2 obtained by Shore and Shore is very similar to that of apoLP-Ala. Several differences in our technique may be noted. Shore and Shore utilized plasma from nonfasting patients and a slightly different technique of delipidation. They also did not employ an initial separation by gel filtration and used a Tris buffer in 8 M urea for the DEAE-cellulose chromatography. We do not know which of these differences in methods might account for the greater heterogeneity of VLD apolipoproteins encountered by Shore and Shore.

Only tentative conclusions can be reached concerning the possible configurations of the VLD apolipoproteins. All of the evidence has thus far been derived from optical studies of the proteins obtained by delipidation. The CD spectrum of apoLP-Ala contains a well resolved minimum at 204 nm suggesting principally random coil structure. A weak band in the 220 to 230 nm region is poorly resolved and overlaps the much stronger neighboring band. Contributions to the ellipticity in this region could be due to a small content of a helix or β structure or might come from the aromatic chromophores of tyrosine and tryptophan that have strong absorption bands in the wave length range (18). The CD and ORD spectra of apoLP-Val suggest a significant content of helical structure. It is of interest that the two major apolipoproteins of high density lipoprotein from the density range 1.125 to 1.195 g per cm^3 contain appreciable quantities of a helical configuration (19). The apoprotein of low density lipoprotein (d = 1.019 to 1.063) appears to contain a mixture of β, α helical, and random coil structure (20).

Polymerase of the apoLP-Ala has been shown to be related to a difference in sialic acid content of roughly 1 mole per mole between the two predominant forms as observed on DEAE-cellulose chromatography and polyacrylamide gel electrophoresis. Similar changes in apparent charge and mobility of protein due to the sialic acid content have been shown for alkaline phosphatase and leucine aminopeptidase in chicken plasma (21).

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