New protein in human blood plasma, rich in proline, with
lipid-binding properties

(apolipoproteins/lipoproteins/chylomicrons)

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ABSTRACT A protein that binds to a lecithin-stabilized triglyceride emulsion has been separated from plasma after removal of major lipoprotein classes by ultracentrifugation at density 1.21 g/ml. This protein, rich in proline, has been purified to electrophoretic and immunochemical homogeneity by subsequent gel and ion-exchange chromatography. In native plasma and after purification, it exists as a large particle exceeding 10^6 daltons, but a single component with a molecular weight of about 74,000 is found upon polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Although the purified protein contains very little bound lipid and is not present in the major lipoprotein classes from post-absorptive individuals, it is present in chylomicrons. Its concentration in plasma varies from 12 to 41 mg/dl and is significantly correlated with that of cholesterol in lipoproteins of very low and low density but not in those of high density.

Some of the protein species of serum lipoproteins have considerable affinity for emulsified triglycerides. A lecithin-stabilized triglyceride emulsion (Intralipid) binds the C group of apolipoproteins in serum or its 1.006 g/ml density (d; all densities are given in g/ml) infranatant fraction (1). We have observed that other proteins are bound to Intralipid when increasing amounts are mixed with serum. Some of these additional proteins bind when the emulsion particles are incubated with serum from which all of the major lipoprotein classes have been removed by centrifugation at a density of 1.21. Investigation has shown that one of the two major species is not detectable in the major lipoprotein fractions of normal human plasma. In this report, we show that this “proline-rich protein” (PRP) is a distinct plasma protein, heretofore uncharacterized, and present evidence that it may be related functionally to those lipoproteins that contain the B apoprotein.

METHODS

Materials. Plasma from fasting adults was obtained from blood containing disodium EDTA (1 mg/ml). Solid KBr was added to give a non-protein solvent density of 1.21 and lipoproteins were separated by centrifugation for 4.4 × 10^8 g-min in the 60-Ti rotor of a Beckman preparative ultracentrifuge at 4°. The infranatant protein solution, obtained after tube-slicing, was dialyzed thoroughly against 0.15 M NaCl containing 0.045 M CaCl_2. Fibrin formed was removed by filtration. In some experiments, the 1.21 density infranatant fraction was obtained after successive centrifugations at 1.006, 1.063, and 1.125 g/ml (2).

Intralipid triglyceride emulsion, 10%, was from Vitrum AB, Stockholm, Sweden. Antisera against human plasma proteins were from Behring Diagnostics, Somerville, N.J.

Purification of Proteins Bound to Intralipid. The dialyzed 1.21 density infranatant fraction of about 200 ml plasma was mixed with 0.2 volume Intralipid and incubated at 37° for 1 hr. The mixture was then centrifuged for 4.5 × 10^8 g-min. After tube-slicing, the infranatant fraction was incubated with fresh Intralipid and centrifuged as before. The combined supernatant lipid layers were dispersed in about 30 ml of 0.15 M NaCl and subjected to chromatography on a 5 × 95 cm column of 4% agarose gel (Bio-Rad Laboratories, Richmond, Calif.). The purified emulsion particles, uncontaminated by unbound plasma proteins, emerged in the void volume. This material (about 200 ml) was concentrated to 30-50 ml by centrifugation. Lipids were extracted from the suspension into 20 volumes ethanol-diethyl ether (3:1 vol/vol) for 24 hr at 4°. After washing with diethyl ether, the precipitated protein was immediately dissolved in 3 ml of 0.09 M Tris- HCl, pH 8.2, containing 6 M urea and subjected to chromatography on a 1.2 × 95 cm column of 4% agarose gel. Eluates of the main protein peak that emerged shortly after the void volume were pooled and subjected to chromatography on a 1.2 × 20 cm column of DEAE-cellulose (Whatman DE 52, W. & R. Balston Ltd., Maidstone, Kent, England) (3) with a gradient of Tris-HCl, pH 8.2, ranging from 0.09 M to 0.15 M in the presence of 6 M urea.

Analytical Techniques. Gel electrophoresis was performed at pH 8.9 (4). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (5). Isoelectric focusing of 2-3 mg of protein was carried out at 20 V/cm for 48 hr at 4° in column 8101 (110 ml, LKB Produkter AB, Bromma, Sweden) using 1 or 2% Ampholine in 6 M urea. Amino-acid composition was determined on protein hydrolysates (6) with a Beckman 121 M micro-analyzer. Total cholesterol and triglycerides in serum and ultracentrifugally separated very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) were determined by standard methods (7).

Preparation of Antisera and Immunoadsabs. The proteins from gel or ion exchange chromatography were further purified by separation on alkaline 3.75% polyacrylamide gels and visualized under ultraviolet light after staining with 8-anilino-1-naphthalene-sulfonic acid, Mg salt (Eastman Chemical Co., Rochester, N.Y.), 0.0065% in saturated ammonium sulfate, for 15 min. The fluorescent band was cut from the gel and homogenized in a small volume of 0.15 M NaCl. Five to 7 ml of material containing 2-3 mg of protein, obtained from 8-12 gels, was emulsified with 2-3 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.) and injected intradermally at the nape of the neck and upper back of New Zealand albino rabbits (2.5-3.0 kg). The injection was repeated three times at intervals of 2
weeks and the animals were bled 10 days after the last injection. Monovalent antisera to individual human apolipoproteins [R-Gln-I, R-Gln-II, arginine-rich protein, the three "C"-apoproteins (R-Ser, R-Glu, and R-Ala)] (3) and to human LDL (1.024 < d < 1.050) were prepared similarly. Double immunodiffusion (8) was performed in 1% agarose gel prepared in 0.05 M barbital buffer, pH 8.6. Immunoelectrophoresis was carried out in the same medium at 2 mA/cm for 90 min. The precipitin arcs were stained with Amidoschwarz (9). For quantitative radial immunodiffusion (10), 0.5 ml of antiserum was mixed with 100 ml of 1.2% agarose in 0.05 M barbital buffer, pH 8.6 containing 0.02% sodium azide. Plates 1 mm thick were prepared and samples of 5 and 10 μl of serum were applied. Standards containing 0.5–5.0 μg of PRP gave a linear response of area to concentration.

RESULTS

Characterization of Proteins Bound to Intralipid from 1.21 Density Infranatant Fraction of Serum. Upon electrophoresis in 7.5% polyacrylamide gel, the mobility of the major protein band bound to Intralipid was similar to that of R-Gln-I, the major apoprotein of HDL (Fig. 1). In double immunodiffusion the bound proteins gave a strong precipitin line against anti-R-Gln-I serum and a relatively weak line against anti-R-Gln-II serum. In some samples a weak reaction was observed against anti-arginine-rich protein, but never against antisera to R-Ser, R-Glu, or R-Ala. Stainable material was consistently observed at the interface between the upper and lower gels. In 3.75% polyacrylamide, this material migrated in two bands a short distance into the running gel. When the infranatant fraction at density 1.21 was obtained after sequential centrifugation at increasing densities, the bound proteins gave additional bands in polyacrylamide gels in the regions of arginine-rich protein and one or more of the C apoproteins (Fig. 1) and they reacted with C protein antisera in addition to R-Gln-I, R-Gln-II, and arginine-rich protein. For this reason the 1.21 density fraction was routinely obtained by initial centrifugation at this density.

When the bound proteins were subjected to chromatography on 4% agarose gel, 90–95% was recovered in five peaks (Fig. 2). The second peak contained 60–65% of the total protein (judged from light absorption at 280 nm) and consisted primarily of the bands that migrated slowly in 3.75% gels (Fig. 3). From its immunological reactivity (described below) this material, as well as the small amount of material eluted in the void volume (peak 1), appeared to contain primarily a single protein, which we have designated PRP. Peaks 3 and 4 contained mainly R-Gln-I as judged from mobility in polyacrylamide gel and immunoreactivity. The small fifth peak gave no stainable band on polyacrylamide gels and did not react with any of the apolipoprotein antisera.

Characterization of PRP. The material from peak 2 was eluted from DEAE-cellulose columns in two to three overlapping peaks between 0.11 and 0.13 M tris-Cl, and represented 50–65% of the applied protein (Fig. 4). The number of peaks and the elution patterns varied in different samples, but all fractions gave the same pattern on gel electrophoresis, with a major band and a minor one slightly preceding it (Fig. 3). Antisera to these fractions and to whole serum gave single and identical precipitin lines with each fraction. When the columns were eluted with 0.4 M tris-Cl, a small additional amount of protein was obtained that also reacted with antisera to PRP. In sodium dodecyl sulfate–polyacryl-
amide gel electrophoresis, the protein in each of the major peaks gave a single band that corresponded to a molecular weight of 74,000 (Fig. 5). Upon immunoelectrophoresis the precipitin arc of PRP was very close to that of beta lipoprotein but distinct from it (Fig. 6). Because PRP behaved as a large particle, antiserum to LDL, Lp(a), IgM, the Cfq component of complement, C-reactive protein, antihemophilic factor, coagulation factor 13, and alpha-macroglobulin were tested against the purified protein by double immunodiffusion. None showed any reaction. Upon isoelectric focusing with a pH gradient of 3-10, the pooled material from DEAE-cellulose columns had a pI of about 6.4. In a column with a gradient of 5-7, the pI varied from 6.1 to 6.4 and occasionally two bands were evident. PRP was very slightly soluble in water but dissolved readily in 0.05 M Tris-HCl, pH 8.5. Amino-acid analysis (Table 1) showed an unusually large amount of proline and substantial cysteine. PRP, partially purified from 1.21 density infranatant fractions of plasma by chromatography on 4% agarose gel, contained no detectable phospholipids and <4% by weight of neutral lipids.

Distribution of PRP in Serum Protein and Lipoprotein Fractions. When whole serum from normolipemic or hyperlipemic donors was subjected to chromatography on 4% agarose gel, material reacting with anti-PRP emerged shortly after the void volume and just before IgM, as judged from double immunodiffusion of fractions against specific antiserum, and just before LDL, as judged from elution of the major peak of cholesterol (Fig. 7). Highly purified PRP also eluted from 4% agarose columns before purified LDL. However, immunologically reactive PRP was not found in any of the major lipoprotein fractions from normal human serum or from a fraction with \( d < 1.08 \) separated by ultracentrifugation after raising the density with \( D_2O \). The lipoprotein fraction (VLDL + LDL) obtained from human serum by precipitation with heparin and \( MgCl_2 \) or dextran sulfate and

Fig. 6. Immunoelectrophoretograms of proteins in whole serum (added to each well) against antiserum to: (1) whole human serum (rabbit was also given injection of PRP to increase its titer); (2) PRP + LDL + IgM; (3) PRP; (4) same as 1; (5) LDL.

Table 1. Amino-acid composition of PRP

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>(mol/10^3 mol of amino acids)</th>
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<tr>
<td></td>
<td>Mean*</td>
</tr>
<tr>
<td>Lysine</td>
<td>59</td>
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<tr>
<td>Histidine</td>
<td>27</td>
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<tr>
<td>Arginine</td>
<td>51</td>
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<tr>
<td>Aspartic acid</td>
<td>90</td>
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<tr>
<td>Threonine</td>
<td>72</td>
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<tr>
<td>Serine</td>
<td>91</td>
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<td>Glutamic acid</td>
<td>127</td>
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<tr>
<td>Proline</td>
<td>89</td>
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<tr>
<td>Half-cystine</td>
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<tr>
<td>Glycine</td>
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<tr>
<td>Alanine</td>
<td>41</td>
</tr>
<tr>
<td>Valine</td>
<td>46</td>
</tr>
<tr>
<td>Methionine</td>
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</tr>
<tr>
<td>Isoleucine</td>
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<tr>
<td>Leucine</td>
<td>64</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>38</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>38</td>
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</tbody>
</table>

* Three determinations on separate preparations
**Fig. 7.** Elution of hyperlipemic whole human serum from 4% agarose gel. PRP elutes with LDL and IgM; VLDL elutes in the void volume. Immunoreactivity (assayed by double immunodiffusion) of individual fractions is indicated by + for strong reaction or ± for faint reaction.

MnCl₂ failed to react with anti-PRP, whereas the supernatant fraction did. PRP was precipitated from serum 33% saturated with ammonium sulfate. Immunoreactive PRP was detected in a single preparation of chylomicrons purified by ultracentrifugation and chromatography on 2% agarose gel. It was also found in the d < 1.006 serum lipoprotein fraction from one individual with severe mixed hyperlipemia (but not from subjects with endogenous hyperlipemia) and in chylomicrons separated from two patients with endogenous hyperlipemia 4 hr after feeding 100 g of fat.

**Concentration of PRP in Serum.** One hundred one random samples of blood serum collected from fasting employees of an industrial corporation who were participating in a survey of lipoprotein concentration and distribution were subjected to radial immunodiffusion to determine PRP. The mean concentration was 21.7 mg/dl (SD 5.4) and the range was 11.6–40.6. No difference between serum and plasma was observed. The concentration of PRP correlated directly with that of serum cholesterol (Fig. 8) and LDL-cholesterol and to a lesser degree with VLDL-cholesterol and triglycerides and serum triglycerides, but not with HDL-cholesterol (Table 2). Sera from two subjects with genetically determined abetalipoproteinemia contained 16.4 and 8.4 mg/dl.

**PRP in Other Mammals.** Serum from rats, guinea pigs, dogs, goats, and turkeys gave no reaction with anti-PRP in double immunodiffusion. Serum from chimpanzee reacted identically with human PRP. Old world (macaque and langur) and new world (capuchin and night) monkey sera showed partial identity, but sera from prosimians (lemur and lepilemur) failed to react. Proteins bound to Intralipid from infranatant fractions (density 1.21) of rat and rabbit plasma contained little or no material with the characteristics of PRP as judged from elution of the protein from 4% agarose gel and from protein bands obtained in 3.75% polyacrylamide gel.

**DISCUSSION**

Two outstanding properties of the protein described in this report are its large size and affinity for lipid. Although PRP is present in appreciable concentration in normal human plasma, it does not appear to be a previously described species. Among the known plasma proteins, only β₂-glycoprotein contains as much proline; its amino-acid composition is, however, quite different from that of PRP (11). The physical properties and amino-acid composition of PRP are also distinct from those of characterized human apolipoproteins (12, 13).

In gel chromatography PRP, both as it exists in native plasma and following purification, elutes just before LDL, but it evidently contains little or no bound lipid. The presence of PRP in chylomicrons is consistent with the property of binding to Intralipid, the particles of which resemble chylomicrons in size, structure, and stabilization primarily by lecithin. However, we have not detected PRP in VLDL, which are smaller triglyceride-rich particles with a structure resembling that of chylomicrons.

Several observations suggest a possible relationship between PRP and apolipoprotein B. The electrophoretic mobility of PRP is close to that of beta lipoprotein (LDL), the apoprotein of which is almost entirely apo-B. More importantly, its concentration is significantly related to that of LDL total cholesterol and, to a lesser extent, that of VLDL total cholesterol and triglycerides. VLDL and LDL share

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**Table 2.** Correlation between PRP and lipids in serum and lipoproteins

<table>
<thead>
<tr>
<th></th>
<th>mean ± SD (mg/dl)</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum total cholesterol</td>
<td>238 ± 48</td>
<td>0.534</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum triglyceride</td>
<td>146 ± 92</td>
<td>0.353</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VLDL triglyceride</td>
<td>94 ± 89</td>
<td>0.294</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>LDL total cholesterol</td>
<td>152 ± 50</td>
<td>0.469</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL total cholesterol</td>
<td>57 ± 18</td>
<td>-0.157</td>
<td>NS</td>
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</tbody>
</table>

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Fig. 8. Relation between concentration of PRP, determined by radial immunodiffusion, and concentration of serum total cholesterol (TC). Closed symbols = men and open symbols = women. The correlation coefficient (r) of 0.534 is significant at the 0.1% level.
apo-B. By contrast, the concentration of PRP is not related to that of HDL-cholesterol, which shares other apoproteins (primarily the C apolipoproteins) with VLDL, but not with LDL. Thus, by inference, the concentration of PRP must be related to that of apo-B, and is probably not related to that of the C apolipoproteins. Apolipoprotein B has not been well characterized, but when delipidated, it exists as a large aggregate, as does PRP. Apo-B may contain subunit species (14) and it is possible that PRP represents one of these. However, the amino-acid composition of PRP differs sufficiently from that of apo-B that it cannot represent a major part of the apo-B complex.

The other lipoprotein in normal plasma that contains apo-B is Lp(a). Lp(a) also contains a specific apoprotein with 7.2 mole % of proline (15), but its amino-acid composition differs substantially from that of PRP.

Lees has reported that a β-globulin is present in the 1.21 density infranatant fraction of human plasma, antibodies to which recognize arsanilated or acetylated beta lipoprotein, but not native LDL (16). Like PRP, it is present in chylomicrons (but not in other plasma lipoproteins) and in plasma of patients with abetalipoproteinemia. We have prepared arsanilated and acetylated LDL and have not found that they react with antisera to PRP. Therefore, a relationship of PRP to the protein characterized immunologically by Lees remains uncertain.

The basis for the apparent multimeric state of PRP in solution is not evident. Our limited studies to date suggest that this property is not the result of binding to lipid and that the subunits have close to the same mass. The function of PRP is obscure. Its possible relationship to apo-B remains an attractive subject for investigation.

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proteinemia, and Vincent M. Sarich and Allan C. Wilson for sera from primates. J.P.K. is an Established Investigator of the American Heart Association. This research was supported by USPHS Grant HL-14237.

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