Proteolysis of Apoprotein B during the Transfer of Very Low Density Lipoprotein from Hens’ Blood to Egg Yolk*

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We report an example of the enzymic cleavage of an apoprotein B (apoB), the main apoprotein in the very low density lipoprotein (VLDL) of laying hens’ blood, in a normal biological process, the formation of egg yolk. Plasma VLDL was labeled in vivo with 3H-amino acids, isolated by centrifuging, and injected into another laying hen. Yolk VLDL was isolated and its apoproteins were separated. ApoB was not detected in this lipoprotein. Most of the label originally in apoB was distributed among four smaller yolk apoproteins, apovitellenins III to VI, which are a large proportion of the apoproteins of VLDL in yolk. This distribution of 3H suggested that 80% of apoB was cleaved at three places. One yolk apoprotein, apovitellenin II, was not labeled, indicating that it did not originate from an apoprotein in plasma VLDL. The site for cleavage of apoB in the ovarian tissue has not been determined, but cleavage may occur during receptor-mediated endocytosis. The pattern of cleavage of apoB during transfer to yolk was not imitated by some known proteolytic enzymes.

The proteolysis of apoB, a major apoprotein in blood lipoproteins, is of increasing interest (1, 2). This proteolysis may have particular significance for the study of arteriosclerosis, since products of enzymatic breakdown of apoB have been detected in lipoprotein fractions extracted from pathological human aortas, and these fragments may regulate the rate of cholesterol deposition in situ (3). Although apoB is plentiful in the blood lipoproteins of laying birds, it does not occur in the lipoproteins of egg yolk. In fact, the only apoprotein from blood lipoproteins to be transferred unchanged to yolk in large amount is apovLDL-II (4). In yolk this apoprotein is referred to as apovitellenin I (4). Electron microscopy shows that particles of VLDL in hens’ blood pass apparently unchanged directly into yolk by receptor-mediated endocytosis at the surface of the oocyte (5–7) and during this process it is likely that core triacylglycerides of the VLDL remain intact. There is also immunological evidence for the transfer of apoproteins of blood VLDL to yolk (8, 9).

To explain the absence of the main blood apoprotein from yolk, it was suggested that the principal apoprotein of VLDL, apoB, is split enzymatically to give smaller yolk apoproteins (10). We have now tested this suggestion by determining the fate of 3H-labeled apoB after its passage into yolk. We have verified that apoB is degraded although, in contrast to the earlier suggestion (10), we have found that all the high molecular weight apoproteins (M₄ > 6 X 10⁶) of the yolk lipoprotein were derived from apoB.

Some aspects of this work have been reported in abstract form (11).

EXPERIMENTAL PROCEDURES

Materials—Australorp hens, less than 1-year-old from Line 65 produced by CSIRO Division of Animal Production, North Ryde, NSW, were used.

3H-Labeled amino acids were from Amersham Australia Pty Ltd., Sydney, NSW, and had the following levels of specific activity, in GBq/mg: L-[2,3-3H]alanine, 16–24; L-[3,5-3H]lysine, 9; [2-3H]glycine, 5–7; L-[4,5-3H]lysine monohydrochloride, 15–17; and L-[4,5-3H]leucine, 13.

Of the proteolytic enzymes used, porcine kallikrein, trypsin, and chymotrypsin, were from Sigma, human thrombin, from the Commonwealth Serum Laboratories, Melbourne, Victoria.

Preparation of 3H-Labeled VLDL—Radioactively labeled VLDL was prepared by injecting into the wing vein of a laying hen the following mixture of 3H-labeled amino acids: glycine, lysine, alanine, tyrosine, and leucine dissolved in 0.16 M sodium chloride in a volume not exceeding 5 ml and containing a total amount of radioactivity not greater than 170 MBq. The mixture contained either an equal amount of activity from each amino acid or, on some occasions, a 5-fold greater level of [3H]leucine than any single amino acid. There was no difference between the results obtained using these two mixtures.

Preliminary tests showed that maximum incorporation of label into VLDL occurred within 2.5 h. Blood, therefore, was collected from birds anesthetized with sodium pentobarbitone by section of the jugular vein between 2 and 2.5 h after injection. EDTA was used as an anticoagulant (final concentration 5 mg/ml of blood) and plasma was separated by centrifugation.

VLDL was isolated by mixing the plasma with an equal volume of 2 M sodium chloride (final density 1.03 g/ml) containing cysteine (1 mM) and centrifuging on a Beckman L-8 preparative ultracentrifuge for 2 × 10⁵ h g at 3°C. At the density used, the lipoprotein floated to form a compact layer that was easily removed. It is known that LDL, which would have been included, is a very small proportion of the total lipoprotein in laying hens’ blood (10). The floating lipoprotein was redispersed and recentrifuged under the same conditions. The absence of albumin and other plasma proteins was shown by gel electrophoresis, which also confirmed the presence of apoB and apovLDL-II as the main apoproteins in VLDL.

In Vivo Transfer of 3H-labeled Plasma VLDL into Yolk and Isolation of VLDL and Other Yolk Constituents—Labeled VLDL in 0.16 M sodium chloride (2.5 to 5 ml; 4 to 22 × 10⁶ dpm) was injected into the wing vein of another laying hen. Eggs laid up to 8 days after injection were monitored for 3H activity. Maximum radioactivity was found in yolks of eggs laid 3 to 6 days after injection and eggs were collected during this period for analysis.

The major yolk lipoprotein was isolated as follows. The yolk was diluted with an equal volume of 0.16 M sodium chloride and centrifuged (1 × 10⁵ h g at 10°C) to sediment the yolk granules (12). The supernatant solution was diluted with an equal volume of 2 M sodium chloride and the mixture was centrifuged as for plasma VLDL.

The yolk granules were freed from VLDL by dissolving them in 2 M sodium chloride and by centrifuging using the conditions for preparation of VLDL. The yolk levine fraction was obtained from the supernatant solution remaining after collection of VLDL. This was recentrifuged under the same conditions used for VLDL and

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† The abbreviations used are: apoB, apoprotein B; VLDL, very low density lipoproteins; LDL, low density lipoproteins; SDS, sodium dodecyl sulfate.
residual VLDL was removed before the sample was mixed. Finally, an aliquot was taken for measurement of radioactivity.

There was no evidence for proteolytic activity against apoB in yolk. This was initially tested by mixing plasma VLDL with whole yolk and demonstrating the continued presence of apoB in the mixture. Subsequently, 3H-labeled plasma VLDL was incubated with whole yolk and/or ovarian follicles at pH 5.3-8.6 but no breakdown of apoB was detected.

**Isolation of Labeled VLDL Apoproteins**—Lipid was removed and the apoproteins were isolated from yolk and plasma VLDL by procedures described previously (10). For electrophoresis the plasma VLDL apoproteins were dissolved in 6 M urea, 2% SDS. The 3H activity of the total isolated lipid was measured and the lipids were separated into classes by thin layer chromatography (13).

**Column Chromatography**—For gel filtration chromatography, columns of Sepharose CL-6B (Pharmacia P-L Biochemicals) were used with 6 M urea, 0.02 M HCl, pH 3.3, as solvent under previously described conditions (14). The yolk apoproteins, apovitellenin I, IV, and VI, were isolated by gel filtration and hydrophobic chromatography as described previously (15). Their purity was checked by gel electrophoresis.

**Gel Electrophoresis**—Electrophoresis was performed on polyacrylamide gels essentially following the procedure of Weber and Osborn (16) with modifications as described below. For analytical purposes, tube gels (7.5 × 0.5 cm) containing 4% polyacrylamide were used, with bisacrylamide cross-linking. For larger scale separations, which were used to determine the 3H activity in apoproteins, large gels (15 × 14 × 0.15 cm) were used with N, N'-(1,2-di hydroxyethyl) bisacrylamide as cross-linker as described by O'Connell and Brady (17). This cross-linker was used so that the gel could be dissolved under mild conditions for subsequent radioactive counting. After electrophoresis for 2 h at 8 V/cm, the gels were stained lightly with Coomassie Blue. The stained protein bands, the origin, and unstained zones were then cut out and dissolved by immersion in 130 mM periodic acid for 24 h at 50 °C. Aliquots of this mixture were taken for measurement of 3H activity.

**Measurement of Radioactivity**—Aqueous samples were suspended in a gel scintillation mixture, containing the solvents water, toluene, and teric XII (ICI, Australia Ltd.) in the proportion 2.5:3:4 and phosphor solutes 2.5-diphenyloxazole (4 g/l) and 1.4-bis [2-(5-phenyloxazoyl)] benzene (0.1 g/l). Lipids were dissolved directly in a toluene-based scintillation mixture. Radioactivity was measured using a Packard Tri-Carb 2660 liquid scintillation system where quenching was measured with the external standard channels-ratio method and checked by the use of an internal standard. Specific radioactivity of freeze-dried proteins from column fractions was measured on a few samples using a manual combustion method (18).

**Proteolytic Digestion of VLDL in Vitro**—A solution of VLDL in 20 mM Tris buffer, pH 7.4, was incubated with the proteolytic enzyme at 37 °C. At intervals small samples (50 µl) were added to an equal volume of 4% SDS and the mixture was either applied to an electrophoretic gel or frozen and subsequently electrophoresed. From 10 to 20 µg of isolated apoprotein was applied to rod gels as described under "Experimental Procedures." Gel a, apoproteins from plasma VLDL; Gel b, apoproteins from egg yolk VLDL; Gel c, apoproteins from plasma VLDL after digestion for 1 h at 37 °C with kallikrein as described under "Experimental Procedures." For Gel b the Roman numerals refer to apovitellenins I to VI with minor bands designated by letters (e.g. IIIa). The scale of molecular sizes refers to gel b. The lower right-hand arrowheads denote the positions of the marker dye. Electrophoresis was from top to bottom.

**RESULTS**

**Apolipoprotein Patterns of Plasma and Yolk VLDL**—The gel electrophoretic patterns in Fig. 1 illustrate the large differences in VLDL apoproteins between the plasma of laying hen's blood and egg yolk. These are that there is no apoB in yolk, but a range of smaller apoproteins, the apovitellenins II to VI, and several minor constituents. One apoprotein is common to VLDL from plasma and yolk. The amino acid sequence of this protein is identical in plasma, where it is named apovLDL-II, and in yolk, where it is named apovitellenin 1 (4).

**Distribution of 3H Radioactivity in Plasma VLDL Labeled in Vivo**—Incorporation of 3H in vivo (see "Experimental Procedures") provided a plasma VLDL preparation in which more than 95% of the total radioactivity in plasma VLDL apoprotein was present in apoB and apovLDL-II. Fig. 2 shows the incorporation of 3H into VLDL apoproteins from one plasma sample separated by column chromatography. In this...
preparation apoB and apoVLDL-II accounted for 71.9 and 23.7%, respectively, of the protein-bound radioactivity, with only 4.4% in minor apoproteins. Separation of three other plasma preparations on polyacrylamide slab gels gave a mean distribution of 76.7 ± 4.0, 19.0 ± 6.2, and 4.3 ± 2.3% for apoB, apoVLDL-II, and minor apoproteins, respectively, with S.E. values indicating that there can be variability in this distribution. Only 4.6 ± 0.6% (mean ± S.E. of four samples) of total radioactivity in plasma VLDL was found in the lipid and, of this, 73% was in triacylglyceride and 21% in phospholipid.

Distribution of \(^3\)H Radioactivity in Yolk VLDL—Of the radioactivity injected as plasma VLDL into recipient hens, 37 ± 4% (mean ± S.E. for three experiments) was recovered in the yolks of eggs laid up to 8 days following the injection. Of the three yolk-protein-containing fractions, the yolk granules and lipovitellins (see "Experimental Procedures") contained only small proportions (0.9 and 3.7%, respectively) of the radioactivity in comparison with VLDL. This indicated that recycling of \(^3\)H-amino-acids from VLDL made only a minor contribution to yolk protein synthesis and that there was negligible transfer of degradation products from VLDL into these fractions. Other evidence against the resynthesis of labeled amino acids into yolk apoproteins is mentioned below, namely one apoprotein was without detectable radioactivity. Yolk VLDL apoproteins were separated by column chromatography or electrophoresis on gel slabs, but only the latter procedure gave acceptable resolution. Although quantitative separations were not achieved on columns, pure samples of apovitellenins I, IV, and VI were isolated by rechromatography (see "Experimental Procedures"). Measurements on these samples showed that the specific radioactivities of apovitellenins I, IV, and VI were similar to that for apovitellenin I. The distribution of radioactivity between yolk VLDL apoproteins was compared with that of yolk VLDL apoproteins in Fig. 3. This pattern (Fig. 3b) consists predominantly of a band in the position of apoB. There are also bands in the positions of the yolk apoproteins, apovitellenins IV and VI. The proportions of these apovitellenins were reduced by repeated washing of the preparation, suggesting that their presence was due to contamination with yolk and not to proteolysis of apoB in the basal lamina.

**TABLE I**

**Distribution of \(^3\)H in the apoproteins of egg yolk VLDL.**

The apoprotein solution (0.5 to 1.5 ml, 5 to 30 \(\times 10^5\) dpm) was separated by electrophoresis on slab gels and bands cut out for measurement of radioactivity. Details of procedures are given under “Experimental Procedures.”

| Apoprotein (apovitellenin) | \(^3\)H radioactivity (A) | \(M^*\) | \((M/A) \times 10^8\) |
|---------------------------|-------------------------|--------|---------------------|
|                           | % total                 |        |                     |
| VI                        | 25.6 ± 2.1              | 170,000| 6.64                |
| Va + Vb                  | 6.4 ± 0.7               | 120,000| 18.75               |
| V                         | 2.4 ± 0.7               | 50,000 | 41.67               |
| IV + IIIB                | 12.1 ± 0.9              | 25,000 | 6.87                |
| III                      | 17.2 ± 1.4              | 75,000 | 4.36                |
| II                       | 10.6 ± 1.0              | 65,000 | 6.13                |
| I                        | 18.1 ± 0.4              | 20,000 | 9.50                |

* Molecular weights quoted from Ref. 14.

Values are mean ± S.E. from five analyses. Radioactivity at the origin was 2.0 ± 1.1% and the remainder was not associated with defined protein bands.

Fractions of similar molecular weight which were not sufficiently separated on slab gels to count as separate bands.

In Vitro Cleavage of Plasma VLDL ApoB—Attempts at detecting cleavage of plasma VLDL apoB in vitro using ovarian tissue preparations from laying hens were unsuccessful. The patterns of cleavage obtained from incubation of plasma VLDL with porcine kallikrein are shown in Fig. 1. Thrombin, chymotrypsin, and trypsin produced substantial breakdown, but with few clearly distinguishable fractions of molecular weight greater than 30,000.

**Apoproteins of Plasma VLDL in the Basal Lamina of the Granulosa Cell Layer**—The basal lamina represents the first extravascular barrier to the transfer of plasma VLDL from the bloodstream to the interior of the oocyte (19). VLDL was collected from pooled basal lamina and the apoproteins were separated by electrophoresis. A densitometric scan of a gel of these apoproteins is compared with that of yolk VLDL apoproteins in Fig. 3. This pattern (Fig. 3b) consists predominantly of a band in the position of apoB. There are also bands in the positions of the yolk apoproteins, apovitellenins IV and VI. The proportions of these apovitellenins were reduced by repeated washing of the preparation, suggesting that their presence was due to contamination with yolk and not to proteolysis of apoB in the basal lamina.

**DISCUSSION**

In the laying hen, VLDL apoB is synthesized in the liver. Many properties of hens' apoB are comparable to those of a protein now referred to as apoB-100 in human blood (20, 21), thus suggesting the possible use of hens' apoB as a model. In the above experiments we have confirmed an unusual feature of avian apoB metabolism, namely its proteolysis during yolk formation. Our evidence is based on the redistribution of \(^3\)H radioactivity when \(^3\)H-labeled plasma VLDL is injected into laying hens and is transferred to the yolk (Table I). ApoB was the only source of label for the yolk apoproteins in these experiments as the other \(^3\)H-labeled apoprotein, apoVLDL-II, is known to be transferred to yolk unchanged in amino acid sequence (4). That proteolysis of apoB does not take place during the isolation of the yolk apoproteins was inferred from the lack of an effect when protease inhibitors are present during isolation (14). Furthermore, after addition of labeled VLDL to yolk no signs of proteolysis of labeled apoB were found. It was originally suggested that apoB is cleaved enzymically to give two fractions (10). It is now clear, however, that this suggestion underestimated the extent of cleavage because Table I shows that all the yolk apoproteins with \(M^*\) greater than 60,000 were labeled. As the main apoproteins are known

![Fig. 3. Densitometric traces of gel electrophoretic patterns. ApoB isolated from VLDL of yolk (a) or basal lamina of the granulosa cell layer (b) were separated on rod gels containing SDS. Details are given under “Experimental Procedures.”](attachment:image_url)
to have a similar amino acid composition (14) and on the assumption of uniform labeling of apoB and specific enzymic cleavage without loss of smaller peptides, the radioactivity of these apoB fragments should be proportional to their size. Table I shows that for apovitellenins III, V, and VI the ratio of \( M_i \) to radioactivity is approximately constant. For apovitellenin IV the ratio of \( M_i \) to activity is lower than for the other three but this could be accounted for by the presence of a minor band, apovitellenin IIIa, that could not be separated by gel slab electrophoresis.

We, therefore, suggest that proteolysis occurs in three places to give four fragments, apovitellenins III–VI, whose total \( M_i \) is approximately \( 4 \times 10^6 \). Values of \( 3.5 \times 10^6 \) have been reported for hens’ apoB (20, 22).

According to Table I, less than 20% of the activity derived from apoB was not present in apovitellenins III–VI but was associated with minor proteins that have not yet been isolated. This suggests either that the enzyme system is not entirely specific in its action on apoB or that there is more than one species of apoB with different amino acid sequences in the regions recognized by the enzyme system. Evidence for the heterogeneity of mammalian apoB has been discussed by Kane (21) and others (23).

We have not been successful in imitating the action of the hens’ enzyme system on VLDL, by using the enzymes kallikrein, thrombin, chymotrypsin, and trypsin. Of these, only kallikrein produced a specific pattern of cleavage with some slight similarity to the natural pattern (Fig. 1, b and c), but it was clearly different. For human apoB-100, human kallikrein produces cleavage at a single site to produce apoB-74 and apoB-26 (24). Although an avian kallikrein has been identified on entering the egg yolk (26). There is no information on the presence of unmodified plasma VLDL in the basal lamina, although it has been known for some time that another large precursor protein in the blood of laying animals, vitellogenin, is cleaved enzymically on entering the egg yolk (28). There is no information on whether the same enzyme system is involved in splitting apoB.

The fact that the second low molecular weight apoprotein in yolk lipoprotein, apovitellenin II, was unlabeled (Table I) shows that this protein does not enter the yolk via VLDL. It also confirms that there was no measurable degradation of VLDL apoproteins and redistribution of the label by recombination of others. A similar conclusion was reached from the low \( ^3\)H activity in the livetin and granule fractions of yolk and the albumen proteins of the eggs examined. Apovitellenin II is unusual in that it is soluble in salt solutions and is also present in the aqueous phase of yolk (27, 28). We have not been able to detect its role in laying hens’ blood serum. We, therefore, offer the speculation that it is part of a membrane protein that is split off during the passage of VLDL through the oolema and that possibly it is part of the receptor itself.

We have presented evidence for proteolysis in vivo as part of a natural metabolic function of apoB. The reasons for this proteolysis are not clear but they could be associated with the vastly different environments of blood and yolk (15). Thus, the egg yolk lipoprotein has to remain stable for days or weeks at atmospheric temperatures in equilibrium with oxygen; whereas in the blood there are reducing conditions and the lipoproteins have a rapid rate of turnover.

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REFERENCES

1. Cardin, A. D., Witt, K. R., Chao, J., Margolias, H. S., and Donaldson, V. H. (1984) J. Biol. Chem. 259, 8522–8538
2. Chung, B. H., Im, J. H., and Bowdon, H. R. (1986) J. Biol. Chem. 261, 2960–2967
3. Clevelend, B. A., Morton, R. E., West, G., Dusek, D. M., and Hoff, H. F. (1984) Arteriosclerosis 4, 196–207
4. Dugaiczky, A., Ingis, A. S., Strike, P. M., Burley, R. W., Beattie, W. G., and Chan, L. (1981) Gene (Amst.) 14, 175–182
5. Perry, M. M., Gilbert, A. B., and Evans, A. J. (1978) J. Anat. 125, 481–497
6. Perry, M. M., and Gilbert, A. B. (1979) J. Cell Sci. 39, 257–272
7. Perry, M. M., Griffin, H. D., and Gilbert, A. B. (1984) Exp. Cell Res. 151, 443–446
8. Hillyard, L. A., White, H. M., and Pangburn, S. A. (1972) Biochemistry 11, 511–518
9. Heal, P. J., and McLachlan, P. M. (1963) Biochem. J. 87, 571–576
10. Burley, R. W., Sleight, R. W., and Shenstone, F. S. (1984) Eur. J. Biochem. 142, 171–176
11. Evans, A. J., and Burley, R. W. (1986) Proc. Aust. Biochem. Soc. 18, 12
12. Burley, R. W., and Cook, W. H. (1961) Can. J. Biochem. Physiol. 39, 1296–1307
13. Mangold, H. K. (1969) in Thin Layer Chromatography (Stahl, E., ed) pp. 375–376, Springer-Verlag, New York
14. Burley, R. W., and Sleight, R. W. (1980) Aust. J. Biol. Sci. 33, 255–268
15. Burley, R. W., and Sleight, R. W. (1983) Biochem. J. 209, 143–150
16. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
17. O’Connell, P. B. H., and Brady, C. J. (1976) Anal. Biochem. 76, 63–73
18. Kalbener, F., and Rutschmann, J. (1961) Helv. Chim. Acta 44, 1956–1966
19. Evans, A. J., Perry, M. M., and Gilbert, A. B. (1979) Biochim. Biophys. Acta 573, 184–195
20. Chapman, M. J., Goldstein, S., and Laudat, M. H. (1977) Biochemistry 16, 3006–3015
21. Kalbener, F., and Rutschmann, J. (1961) Helv. Chim. Acta 44, 1956–1966
22. Williams, D. L. (1979) Biochemistry 18, 1056–1063
23. Young, S. G., Bertles, S. J., Scott, T. M., Dubois, B. W., Curtiss, L. K., and Wittum, J. L. (1986) J. Biol. Chem. 261, 2995–2998
24. Hardman, D. A., Gustafson, A., Schilling, J. W., Donaldson, V. H., and Kane, J. P. (1986) Biochem. Biophys. Res. Commun. 137, 821–825
25. Schleuning, W., Sudol, M., and Reich, E. (1983) J. Biol. Chem. 258, 14106–14114
26. Wallace, R. A. (1985) in Development Biology (Browder, L. W., ed) Vol. 1, pp. 127–177, Plenum Publishing Corp., New York
27. Burley, R. W. (1975) Aust. J. Biochem. 28, 121–132
28. Bengtsson, G., Marklund, S. E., and Olivecrona, T. (1977) Eur. J. Biochem. 79, 211–223