Purification, Characterization, and Conformational Analysis of Rabbit Plasma Lipid Transfer Protein†

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ABSTRACT: A procedure for rapid isolation of lipid transfer protein (LTP) from commercially available rabbit plasma is described. Use of protease inhibitors was important for obtaining intact, stable LTP. After lipoproteins were precipitated from the plasma by dextran sulfate, column chromatographies through Butyl-Toyopearl 650M, CM-Toyopearl 650M, and Butyl-Toyopearl 650M were employed. Overall purification from plasma was (3830 ± 710)-fold with a yield of 3–5%. The isolated LTP migrated as a single band during sodium dodecyl sulfate–polyacrylamide gel electrophoresis with M_r = 74K and had an NH_2-terminal amino acid sequence and amino acid composition closely matching those predicted by its cDNA. This band was recognized by immunoblotting with an anti-human LTP monoclonal antibody, TP2. Gel permeation chromatography revealed that LTP behaved as a globular protein of M_r = 83K. Isoelectric focusing of the isolated LTP demonstrated a ladder of bands with p*I*'s of 5.7–5.9. The specific activity of rabbit LTP was similar to that of human LTP. Monoclonal antibody TP2, that blocked human plasma LTP activity almost completely, only partially inhibited purified rabbit LTP, and rabbit plasma LTP activity to a similar extent. By a centrifugation binding assay, rabbit LTP was shown to predominantly associate with lipid microemulsion in its presence. Circular dichroism spectroscopy indicated a high content of β structure, and Provencher and Glöckner analysis gave estimated fractional values of 0.30, 0.39, 0.12, and 0.19 for α-helix, β-sheet, β-turn, and remainder content, respectively. Upon lipid binding, the helical content did not change drastically, although there was some disordering of β structure.

Rabbit has been a popular model for investigating atherosclerosis because of its susceptibility to diet-induced hypercholesterolemia (Velican & Velican, 1989) and the existence of a rabbit model of familial hypercholesterolemia, one of the most common genetic disorders in man (Van Lenten, 1989). An early survey demonstrated that lipid transfer activity varies greatly among species, with man and rabbit being mammals with significant levels (Ha & Barter, 1982). The ability to mediate net transfer of cholesteryl ester (CE)[1] from high-density lipoprotein (HDL) to triglyceride-rich lipoproteins implicates lipid transfer protein (LTP) in hypothetical reverse cholesterol transport as well as potentially in the regulation of HDL level (Tall, 1990; McPherson & Marcel, 1991). There is wide epidemiological evidence in man that HDL is negatively associated with atherosclerotic heart disease (Gordon & Rifkind, 1989), and a negative correlation between species LTP activity and resistance to diet-induced atherosclerosis has been noted (Stein et al., 1990). From transgenic LTP experiments in mice, LTP in vivo has significant lowering effects on HDL (Agellon et al., 1991; Marotti et al., 1992), while genetic LTP deficiency in man is associated with elevated HDL cholesterol and the absence of heart disease (Inazu et al., 1990). In man, LTP is the sole protein responsible for neutral lipid transfer (Hesler et al., 1988), and the predicted protein sequence of the rabbit version has been found to be extremely similar (Nagashima et al., 1988). Thus, by having LTP, rabbit continues to offer a good animal model of human lipoprotein metabolism as well as a good model to evaluate the role of LTP in lipoprotein metabolism (Abbey & Calvert, 1989; Whitlock et al., 1989).

The basic mechanism of the LTP reaction is not well understood, and at least two models, carrier (Barter & Jones, 1980) and ternary complex (Ihm et al., 1982), have been suggested. Employment of impure fractions seemed to suffice in these kinetic studies, as well as in investigations of the regulation of the LTP reaction by free fatty acid (Sammett & Tall, 1985; Lagrost & Barter, 1991), free cholesterol (Morton, 1988), apolipoproteins (Sparks et al., 1991; Milner et al., 1991), and prostaglandins (Muzya et al., 1987). However, further understanding of the regulation of LTP rests upon elucidation of the basic mechanism of LTP action. Investigation of structure–function relationships based on monitoring the LTP protein conformation with regard to aspects of lipid interaction may lead to further insight in the above areas; however, such types of studies are best done with pure transfer protein.

Thus, we set out to purify rabbit LTP from commercial plasma, a good source for many reasons. First, for convenience, there is the high level of activity in this species (Ha & Barter, 1982), the ready availability, and the lower health risk level of working with rabbit versus human plasma these days. Also, the high similarity between rabbit (Nagashima et al., 1988)

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[1] Abbreviations: BPI, bactericidal permeability increasing protein; CD, circular dichroism; CE, cholesteryl ester; CM, carboxymethyl; EDTA, ethylenediaminetetraacetic acid; HDL, high-density lipoprotein; LBP, lipopolysaccharide binding protein; LDL, low-density lipoprotein; LTP, lipid transfer protein; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TG, triglyceride.

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and human LTP makes rabbit LTP a useful model for comparison with extensive information available on human LTP. In this study, a rapid and good yielding procedure for isolating LTP was developed, and identity was documented by numerous means. The behavior of rabbit LTP in the presence of lipid microemulsions resembling lipoproteins was also evaluated. Finally, structural analysis of LTP by circular dichroism (CD) spectroscopy was performed, and the effect of lipid binding on LTP protein structure was also determined by this method. We discuss how these results provide insight on the mechanism of the LTP reaction and lay foundations for further characterization of this process.

EXPERIMENTAL PROCEDURES

Materials. Aprotinin (Trasylol) and gentamycin (Garamycin) were from Miles and Schering, respectively. [1α,2α-(N)-^{3}H]Cholesteryl oleate (69.7 mCi/mg) was purchased from Amersham. Cholesteryl (pyren-1-yl)hexanoate and triolein were purchased from Sigma. Egg phosphatidylcholine (PC) was obtained from Avanti Polar Lipids. Dextran sulfate–cellulose (Yokoyama et al., 1984) was kindly provided by Kanegafuchi Chemical Industries, Osaka, and enzymatic total and free cholesterol and triglyceride (TG) assay kits were from Wako Pure Chemicals, Richmond, VA. Frozen rabbit plasma was purchased from Pel-freez, Rogers, AR, and Toyopearl Butyl-650M and CM-650M gels were supplied by Supelco Canada. Ampholytes were from Pharmacia. TP2 monoclonal antibody against human LTP was a generous gift from Dr. Yves Marcel, University of Ottawa Heart Institute (Hesler et al., 1988). All other chemicals and materials were from Sigma or Bio-Rad or as stated below.

Lipoprotein and Apolipoprotein Isolation. All centrifugations were performed at 4 °C. HDL was isolated from fresh human plasma by sequential ultracentrifugal flotation in NaBr ($1.07 < d < 1.21$ g/mL) (Hatch & Lees, 1968). [^{3}H]Cholesteryl oleate labeled low-density lipoprotein (LDL) was prepared essentially as previously described (Nishikawa et al., 1986). Briefly, 300 mL of fresh human blood was collected in 0.1% ethylenediaminetetraacetic acid (EDTA), and plasma was obtained after centrifugation at 2000*g* for 15 min. Sodium azide (0.02%), 5,5′-dithiobis(2-nitrobenzoic acid) (2 mM), aprotinin (20 units/mL), and gentamycin (0.1 mg/mL) were added, and then very low-density lipoproteins were floated and removed by centrifugation at 184000*g* for 21 h in a Ti 70 rotor (Beckman). The remaining plasma fraction was resuspended by stirring 3 h. Vesicles were made from 4.5 mg of PC and 7.2 μg (0.5 mCi) of [^{3}H]cholesteryl oleate dried with N_2, by sonication in 2 mL of LTP buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, and 0.02% sodium azide) on ice for 15 min until clear. The vesicle preparation was incubated with the plasma fraction at 37 °C for 24 h, and then the incubation mixture was centrifuged at 2000*g* for 15 min to remove the precipitant. The radiolabeled LDL was obtained by passing the supernatant through a 50-mL dextran sulfate–cellulose column (1 mL/min) equilibrated in LTP buffer in which the retained LDL was eluted with LTP buffer/0.5 M NaCl after extensive washing with LTP buffer. The LDL peak was pooled visually (yellow) and adjusted to $d = 1.07$ g/mL with NaCl before the labeled LDL was isolated by ultracentrifugal flotation at 400000*g* for 2.5 h in a TL 100.3 rotor (Beckman). Both isolated HDL and radiolabeled LDL were dialyzed in 200 volumes of LTP buffer/1 mM EDTA for 48 h with two changes, and then the total and free cholesterol contents were measured by enzymatic assay kits. A typical preparation of radiolabeled LDL was 17.2 mM in CE (22.8 mM total cholesterol) and labeled to 1800 dpm/nmol, while HDL was 6.4 mM CE (7.9 mM total cholesterol). Lipoproteins were stored in aliquots at −70 °C until use.

Apolipoprotein A-I was isolated from human HDL as previously described (Nishikawa et al., 1988).

Assay of Lipid Transfer Reaction. Lipid transfer activity was quantitated as previously described (Ohnishi et al., 1990), except that the reaction volumes were halved and incubations were carried out for 3 h at 37 °C. Thus, reactions contained 40 and 47.5 μg of cholesterol moiety in HDL and radiolabeled LDL, respectively, in a final volume of 237.5 μL of 6% bovine serum albumin (fatty acid-free, Sigma) in LTP buffer. The reaction was stopped as previously described (Ohnishi et al., 1990), except only 12.5 μL of 5% heparin/1 M $MnCl_2$ was used and 100-μL aliquots of reaction supernatant were counted in 5 mL of ACS (Amersham) with a Beckman LS6000TA scintillation counter. Activity values were determined only from data points which were linear with protein content within the assay, which resulted in less than 18% of the donor label being transferred, while blanks exhibited about 0.04% of the donor label transferred. Under the conditions used to stop the assay, a considerable amount of HDL is also precipitated; however, such conditions were necessary to minimize background counts from radiolabeled LDL. The amount of HDL recovered in the supernatant was reproducible (30%), and activity values have been corrected for HDL recovery.

Alternatively, CE transfer was measured between lipid microemulsions of TG and egg PC (1.3:1 by weight) using pyrene-labeled CE as a probe (Milner et al., 1991; Ohnishi & Yokoyama, 1993). Donor microemulsions containing pyrene-CE (5.3 mol % of core lipid) and acceptor microemulsions without pyrene-lipid, 0.8 and 7.2 μg in PC, respectively, were mixed with 4 μg of human apolipoprotein A-I, and then the reaction was initiated by adding 1 μg of LTP in a final incubation volume of 0.4 mL (LTP concentration was 47 nM). The reaction was monitored as the decrease in the ratio of excimer to monomer fluorescence emission peak intensities at 37 °C in a Hitachi F-2000 fluorescence spectrophotometer equipped with a thermostatic cell holder and a micromagnetic stirring unit. The fractional rate of pyrene-lipid transfer, k/L_1 (L_1 being donor lipid pool size), was calculated according to the previously described equation (Milner et al., 1991).

Purification of Lipid Transfer Protein. Rabbit plasma (1.5 L), which was stored frozen, was thawed in a water bath at 37 °C, and then phenylmethanesulfonyl fluoride (PMSF) (1 mM), leupeptin (2 mM), benzamidine (1 mM), and pepstatin A (1 μM) were added. All the following procedures were performed at 4 °C. This was followed by the addition of $MnCl_2$ (0.2 M) and 96 mL of 10% dextran sulfate (final concentration 0.6%) followed by stirring for 10 min. The supernatant was isolated by centrifugation at 9600*g* for 20 min. Addition of $BaCl_2$ (1%) followed by stirring for 10 min and another spin under the same conditions to remove dextran sulfate further gave lipoprotein-depleted plasma. NaCl (2 M) was added before application to the first column.

The lipoprotein-depleted plasma was loaded onto a Butyl-Toyopearl column (5 cm i.d. × 14 cm) preequilibrated in 2 M NaCl at 5 mL/min (Figure 1A). The column was washed with an equal volume of 50 mM NaCl, 2 mM EDTA, and 1 mM PMSF (position 1). Lipid transfer activity was eluted beginning with an 800-mL gradient of 50 mM NaCl, 2 mM EDTA, and 1 mM PMSF to distilled water (position 2) followed by straight water (position 3). Fifteen-milliliter fractions were collected, and 50-μL aliquots across the protein peak were assayed for lipid transfer activity. The tubes

containing activity were pooled and adjusted to 20 mM sodium citrate by the addition of 0.25 volume of 100 mM sodium citrate (pH 5.5) before application to the next column. In between purifications, the column packing was regenerated by washing with 2 M NaOH and extensive rinsing with water.

A carboxymethyl (CM)-Toyopearl column (2.8 cm i.d. × 20 cm) was preequilibrated with first 100 mM sodium citrate (pH 5.5) and then 20 mM sodium citrate (pH 5.5) until the effluent was pH 5.5. The pooled fraction from the previous step was adjusted to 20 mM sodium citrate (pH 5.5) with a 100 mM stock solution and loaded at 4 mL/min (Figure 1B). Then the column was washed with 20 mM sodium citrate (pH 5.5), 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, and 1 μM pepstatin (position 1) until the UV absorbance returned to near base line. Lipid transfer activity was eluted beginning with a sharp 50-mL gradient of 0–1 M NaCl in 20 mM sodium citrate plus protease inhibitors (position 2) continued by 1 M NaCl buffer (position 3). Ten-milliliter fractions were collected, and 20-μL aliquots (larger amounts of high salt will interfere with precipitation of LDL in the assay) across the protein peak were assayed for lipid transfer activity before the active fractions were pooled. In between purifications, the column packing was washed with 0.1 M NaOH, water, and 0.5 M HCl and extensively rinsed with water.

A small Butyl-Toyopearl column (2.3 cm i.d. × 7 cm) was preequilibrated with 1 M NaCl, 1 mM EDTA, and 1 mM PMSF (equilibration buffer). The pooled activity from the previous step (80 mL) was loaded directly onto the column at 1.5 mL/min (Figure 1C) followed by an equal volume of equilibration buffer (position 1). The column was washed with 150 mM NaCl, 1 mM EDTA, and 1 mM PMSF (position 2) until the UV absorbance returned to near base line and further with 3 column volumes of 1 mM EDTA/1 mM PMSF (position 3) before lipid transfer activity was eluted with distilled water (position 4). Three-milliliter fractions were collected, and 10-μL aliquots across the protein peak were assayed for lipid transfer activity. In between purifications, the column packing was washed as above.

Human plasma LTP was purified according to our previous method (Ohnishi et al., 1990).

Electrophoresis and Immunoblotting. Polyacrylamide gel electrophoresis (PAGE) in 0.5% sodium dodecyl sulfate (SDS) was carried out according to the method of Laemmli (1970) using a 12% acrylamide separating gel in a vertical slab mini-gel apparatus (Atto, Japan). Before application, samples were heated at 90 °C in the presence of 1% SDS and 2.5% mercaptoethanol, and some samples were desalted or concentrated by deoxycholate/trichloroacetic acid precipitation (Peterson, 1983). Protein bands were visualized by staining with 0.2% Coomassie Blue R250 in 40% methanol/10% acetic acid (1 h) and destaining with 10% acetic acid.

Native isoelectric focusing was performed by mini-gel using a 6% total/0.96% cross-linker acrylamide gel containing 0.4% Ampholine 3.5–10, 2% Ampholine 4–6, and 0.1% tetramethylethylenediamine. A 20-mL volume of gel solution was polymerized by degassing and addition of 0.6% ammonium persulfate. Samples were taken up in equal volumes of sample buffer containing 15% glycerol, 0.8% Ampholine 3.5–10, 4% Ampholine 4–6, and 0.04% bromophenol blue. The catholyte (upper) was 20 mM NaOH, and the anolyte (lower) was 10 mM phosphoric acid; focusing was performed at 200-V constant voltage for 1.5 h and then at 400 V for 1.5 h. The pH gradient was measured by cutting gel slices down the length of the gel and soaking them in 10 mM KCl for 1 h before measurement by a pH meter.

Protein bands were detected after isoelectric focusing, and in some cases after SDS–PAGE, by immunoblotting. This was performed by soaking the gel in transfer buffer (25 mM Tris base, 192 mM glycine, and 20% methanol) followed by electrophoretic transfer of proteins to a nitrocellulose membrane (Bio-Rad) for 2 h at 200 mA. The membrane was then blocked for 2 h with 5% milk powder/Tris-buffered saline [TBS: 20 mM Tris (pH 7.4)/0.5 M NaCl] and washed twice with 0.05% Tween–TBS (wash buffer) for 5 min before incubation for 2 h with TP2 antibody diluted 1:500 in 2% milk powder/0.05% Tween–TBS (antibody buffer). The membrane was then washed twice with 0.05% Tween–TBS before incubation for 1 h with horseradish peroxidase-linked sheep anti-mouse immunoglobulin (Amersham) diluted 1:10 000 in antibody buffer. The membrane was then washed twice with antibody buffer, twice with wash buffer, and 3 times with TBS. For fluorography, the membrane was then treated with gene detection reagents 1 and 2 (Amersham) (1:1 mixture) for 1 min before covering in Parafilm and exposing to XAR 5 film (Kodak) which was developed by an automated processor.

Gel Permeation Chromatography. Gel permeation chromatography was performed using a Superose 12 HR10/30 column connected to a standard FPLC (Pharmacia) setup at 4 °C. LTP buffer was the mobile phase, and the flow rate was 0.75 mL/min. When the absorbance at 280 nm was monitored, 200-μL samples of the globular protein molecular weight standards catalase (220K), aldolase (158K), transferrin (81K), albumin (66K), ovalbumin (43K), and soybean trypsin inhibitor (22.1K) were markers to calibrate K_{av} versus log molecular weight. The same volume of pure rabbit LTP was then analyzed with elution monitored by LTP assay of column fractions and immunoblotting after SDS–PAGE.

Protein Sequencing and Amino Acid Analysis. Purified rabbit LTP was sequenced and analyzed for a fee by the Alberta Peptide Institute, University of Alberta. Sequencing was done on an Applied Biosystems 473A sequencer. Approximately 100 pmol of sample in water was applied directly onto a Biobrene-treated and trifluoroacetate-washed glass fiber filter which was then subjected to automated Edman degradation. Degraded PTH-amino acids were analyzed by a built-in reverse-phase high-performance liquid chromatography on-line system.

Amino acid analysis was carried out on aliquots of sample dried down in blast furnace treated glass tubes which were then evacuated and sealed. Hydrolysis was carried out for 24 h at 110 °C in 6 M HCl before analysis against reference amino acids separated by cation-exchange chromatography and detected by postcolumn ninhydrin reaction using a Beckman 6300 analyzer.

Lipid Binding Analysis. Small TG/PC microemulsions (20–30 nm) were prepared as previously described (Tajima et al., 1983). The final material had a TG:PC weight ratio of 1.36:1. Binding of rabbit LTP was assessed by incubating pure LTP in LTP buffer/0.5% albumin (Sigma) with or without microemulsion (65 μg in TG and 48 μg in PC) for 15 min at 37 °C in a final incubation volume of 200 μL. Then the sample was centrifuged at top speed in a TLA 100 rotor (Beckman) at 25 °C for 15 min. After completion of the spin, the bottom 100 μL of solution was removed with a microsyringe immersed from above to the tube bottom. The separated halves were analyzed for LTP content by LTP assay against a linear standard curve of activity versus pure protein, and for lipid by enzymatic TG assay.

CD Spectroscopy. Measurements were carried out on a Jasco J-720 spectropolarimeter at room temperature. The

instrument was calibrated with *d*(+)-10-camphorsulfonic acid at 290.5 and 192 nm and with *d*(−)-pantoyllactone at 219 nm. LTP (72 μg/mL) in 10 mM sodium phosphate (pH 7.4)/150 mM NaCl was placed in a temperature-controlled 0.05-cm cell at 25 °C. Ten scans were performed, and signal due to solvent was subtracted. Spectra were also measured in the presence of PC/TG microemulsions as used for binding studies (above) at a final concentration of 72 μg/mL LTP and 504 μg/mL PC. LTP concentration was determined by amino acid analysis determination of alanine and leucine content for calculation of molar ellipticities according to the equation $[\theta] = \theta_{obs}/10lC$ where $[\theta]$, the molar ellipticity in degrees centimeter squared per decimole, equals θ_{obs}, the observed ellipticity in degrees, divided by 10 times l (path length in centimeters) times C [concentration in milligrams per milliliter divided by 109.65 (109.65 being the mean residue weight of LTP)]. The ellipticity versus wavelength data were analyzed by a computer program (CONTIN version 1.0) based on a method (Provencher & Glöckner, 1981) for prediction of secondary structure from CD spectra against 16 proteins of known structure from X-ray crystallography. No correction for carbohydrate content was included in generation of the CD spectra.

Other Methods. Protein concentrations were routinely determined by the method of Lowry (Lowry et al., 1951) with bovine serum albumin as a standard and by amino acid analysis for the circular dichroism studies. The results from either method were similar.

RESULTS

Purification of Rabbit Lipid Transfer Protein. The elution profile of lipid transfer activity from the Butyl-Toyopearl column is shown in Figure 1A. Activity coincided with the single, major protein peak retained by the column and eluted with water. Figure 1B shows the elution profile from the CM-Toyopearl column in the next step. Once again, lipid transfer activity elutes largely within a single major protein peak. The later eluting peak may not contain protein because it was absent in runs without PMSF or benzamidine in the buffers. Shallowing the gradient did not increase resolution of the peak components, and the lipid transfer activity peak simply broadened. The lipid transfer activity eluted by high salt in the previous step was directly applied onto a small Butyl-Toyopearl column, and almost pure transfer protein was eluted (Figure 1C). The lower salt concentration (1 M NaCl) for column equilibration and of sample application may have contributed to the significant purification achieved by the second Butyl-Toyopearl step. However, the CM-Toyopearl step in between was necessary; otherwise, large amounts of other proteins coeluted with lipid transfer activity in this subsequent step.

Typical results of the entire purification procedure are summarized in Table I. The overall purification was about 3500-fold over plasma with a yield of about 3% in this particular experiment. Recoveries of only about 20% were observed after each Butyl-Toyopearl chromatography step, although activity in lipoprotein-depleted plasma could not be determined due to interference of the assay by this fraction and recovery from plasma may not have been complete. Figure 2 shows the SDS–PAGE profile at each step of the purification procedure, with the final result after the second Butyl-Toyopearl step always being a broad, diffuse band not atypical for a glycoprotein, migrating at about M_r = 74K. A smaller molecular weight contaminant is visible below the LTP band, but it is not associated with LTP activity (Figure 2, lane 1)

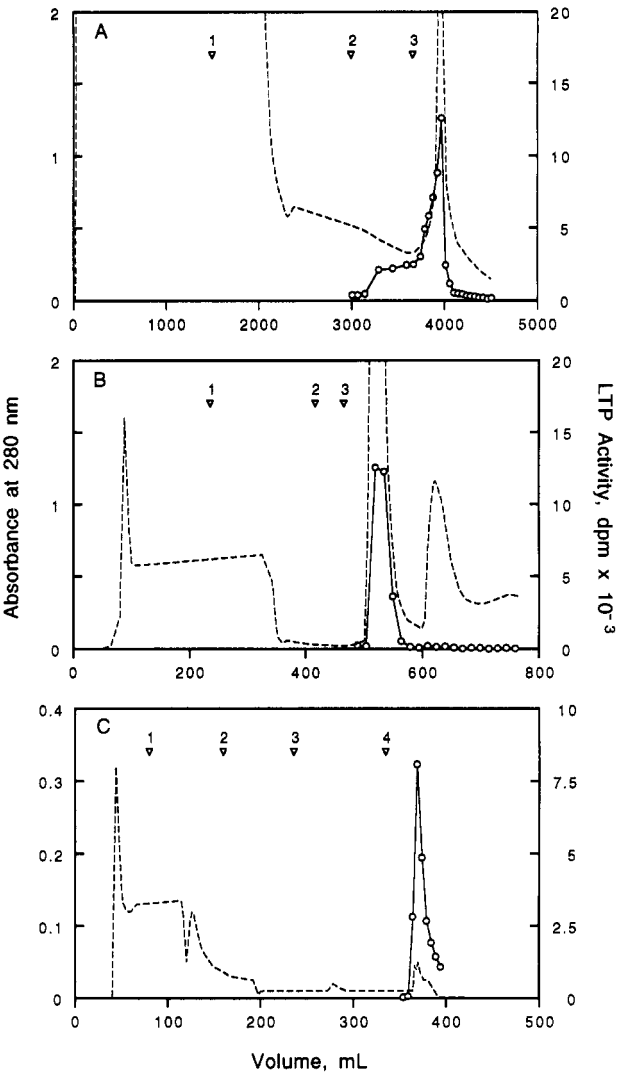

FIGURE 1: Column chromatography during purification of rabbit LTP. (A) Butyl-Toyopearl column; (B) CM-Toyopearl column; (C) small Toyopearl column. Numbers indicate points of buffer changes as described in the text (Experimental Procedures). Dashed line, UV absorbance; open circles, relative LTP activity.

Table I: Purification of Rabbit LTP. Results of a Typical Purification Starting with 1.5 L of Rabbit Plasma

preparation	protein (mg)	act. (nmol/h)	sp act. (nmol h^{-1} mg^{-1})	*x*-fold purification	yield (%)
plasma[a]	84768	302500	3.57	1.0	100
Butyl-Toyopearl	497	61547	123.8	34.7	20.3
CM-Toyopearl	219	42167	192.5	54.0	13.9
Butyl-Toyopearl	0.69	8680	12580	3534	2.9

[a] Data for the lipoprotein-depleted plasma stage (between plasma and the first Butyl-Toyopearl column) were not given because the activity was not linear with protein in our assay system.

and appears to account for less than 5% of the total protein eluted. In three trials, purifications of (3830 ± 710)-fold over plasma with recoveries of 3–5% were reached with essentially the same SDS–PAGE results. If protease inhibitors are omitted from the procedure, similar results are obtained when protein and activity are tabulated, but SDS–PAGE reveals numerous lower molecular weight bands, and the band at M_r = 74K is completely absent. Lipid transfer activity isolated by this procedure is completely stable at 4 °C for at least 3 months.

Characterization of Lipid Transfer Protein. The major band migrating at M_r = 74K was verified as lipid transfer protein immunochemically using TP2, a monoclonal antibody

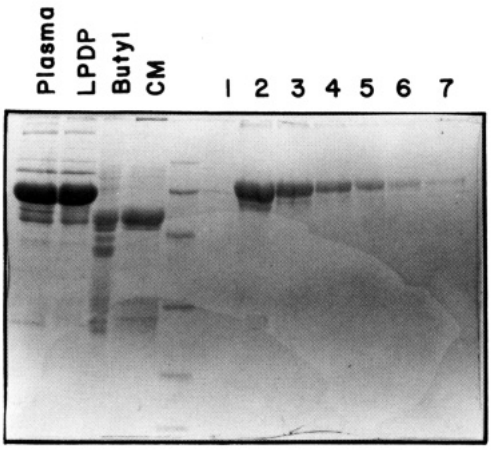

FIGURE 2: SDS–PAGE of protein at each step of the purification procedure. Lanes: Plasma, 10 μg of rabbit plasma; LPDP, 10 μg of protein of LPDP; Butyl, LTP-active fraction from first Butyl-Toyopearl chromatography (40 μg of protein); CM, LTP-active fraction from CM-Toyopearl chromatography (40 μg of protein); lanes 1–7, fractions from small Butyl-Toyopearl chromatography across the LTP activity elution peak (Figure 1C, 200 μL of each). Relative LTP activity in respective fractions is 66, 8077, 4858, 2670, 1916, 1432, and 1080 dpm for these last seven lanes. Bio-Rad molecular weight markers (lane between CM and 1) are (top to bottom) phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme at 97.4K, 66.2K, 42.7K, 31K, 21.5K and 14.4K, respectively.

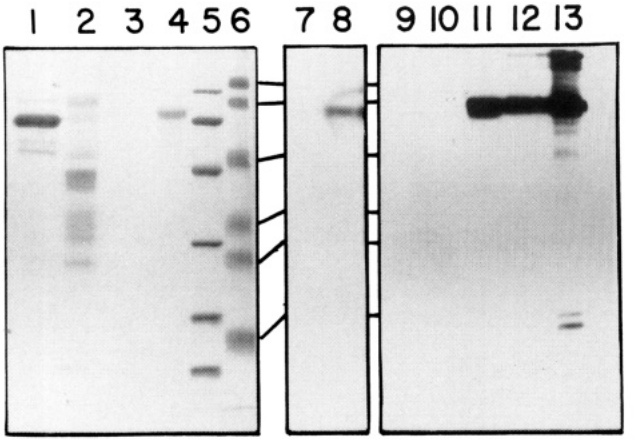

FIGURE 3: Immunoblotting of LTP after SDS–PAGE. Lanes 1–6, Coomassie Blue stained replica gel of various fractions: lane 1, 0.05 μL of rabbit plasma; lane 2, LTP-active fraction from the first Butyl-Toyopearl chromatography (4 μg of protein); lanes 3 and 4, purified LTP fraction after the second Butyl-Toyopearl column, 0.2 and 0.9 μg of protein, respectively. Lanes 7 and 8, immunoblots of plasma, 0.01 and 0.05 μL, respectively, with TP2 monoclonal antibody (3-min exposure). Lanes 9–13, immunoblots of various fractions with TP2 monoclonal antibody (2-s exposure). Lanes 9 and 10 are lanes 7 and 8 (same membrane as above) with a shorter exposure time; lane 11, LTP-active fraction from the first Butyl-Toyopearl chromatography (4 μg of protein, corresponding to lane 2 of the replica gel); lanes 12 and 13, purified LTP, 0.2 and 0.9 μg of protein, respectively, corresponding to lanes 3 and 4 of the replica gel. Lane 5 indicates the migration of molecular weight standards (see Figure 2). Lane 6 indicates the migration of prestained SDS–PAGE low molecular weight standards (Bio-Rad) consisting of (top to bottom) phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme, which behave as proteins of 106K, 80K, 49.5K, 32.5K, 27.5K, and 18.5K, respectively. The angled lines indicate the corresponding positions of the lane 6 markers on the immunoblots, which differ from the replica gel due to shrinkage of the gel during electrophoretic transfer to nitrocellulose.

raised against human LTP and reported to recognize an epitope in the C-terminal region of human LTP involved in lipid binding (Hesler et al., 1988; Swenson et al., 1989). Western blotting and enhanced chemiluminescence detection revealed that this antibody recognized a single protein of M_r = 74K in both crude human (not shown) and rabbit plasma, a partially pure fraction, as well as the single major protein in the purified lipid transfer protein fraction (Figure 3). When the purification was performed in the absence of protease inhibitors, TP2 detects a ladder of smaller molecular weight proteins with many fragments ranging from 50K to 74K and below 18.5K (not shown).

Gel permeation chromatography of purified rabbit LTP (Figure 4) gave a K_{av} of 0.51, corresponding to an M_r of 83K and eluting slightly ahead of albumin, which had a K_{av} of 0.53. Thus, rabbit LTP behaved larger than its predicted protein molecular weight, but similar to results by SDS–PAGE. Other markers and their K_{av}'s were catalase, 0.42; aldolase, 0.45; transferrin, 0.52; ovalbumin, 0.61; and soybean trypsin inhibitor, 0.74.

Native isoelectric focusing revealed a ladder of proteins (about four) migrating with p*I*'s of 5.7–5.9 (Figure 5). Focusing in the presence of 1% mercaptoethanol in the sample buffer lead to slightly further migration. Focusing in the presence of 9 M urea, 2% Triton X-100, and mercaptoethanol lead to a majority of the protein migrating as a smear between pH 6.2 and 7.1 with a small ladder at pH 5.2–5.6 while p*I* standard proteins migrated as normal, discrete bands under the same conditions (not shown). Presumably, denaturing conditions caused lipid transfer protein to aggregate or interact with ampholytes, resulting in anomalous behavior.

Protein sequencing, without chemical modification of cysteines, identified 13 of the first 15 residues from the N-terminal as X-Pro-Lys-Gly-Ala-Ser-Tyr-Glu-Ala-Gly-Ile-Val-X-Arg-Ile-, in agreement with the predicted structure deduced from the cDNA for rabbit LTP (Nagashima et al., 1988), with sequencing yields of 0, 25, 9, 15, 19, 24, 8, 16, 15, 16, 13, 19, 0, 12, and 11% and 2 positions predicted to be cysteines (residues 1 and 13 indicated as X above) giving no

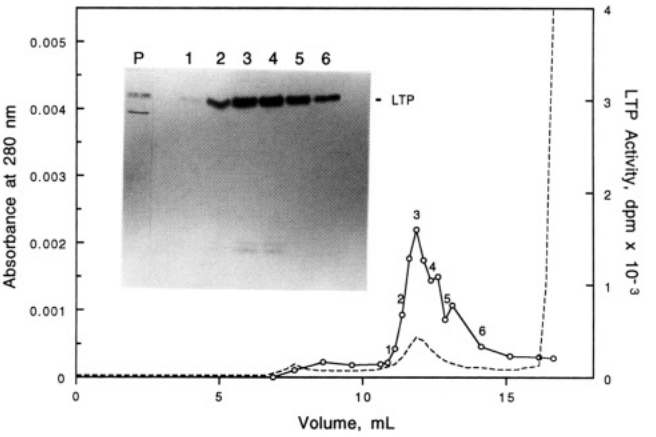

FIGURE 4: Superose 12 gel permeation FPLC of purified rabbit LTP. Dashed line, UV absorbance; open circles, relative LTP activity. V_o was 7.4 mL by elution of blue dextran, and V_t was 16.8 mL by elution of copper salt. Inset: TP2 immunoblots of active fractions (200 μL) eluting at the indicated positions as numbered; P, rabbit plasma (0.1 μL).

signal during the sequencing run. Analysis of the amino acid composition of purified rabbit LTP provided further evidence of its purity. The abundance of determined residues (Asx, Thr, Ser, Glx, Gly, Ala, Val, Met, Ile, Leu, Tyr, Phe, His, Lys, Arg) matched closely the predicted composition of rabbit LTP based on its cDNA sequence, with the only major disagreement being in the number of tyrosines (4.0 molar % of among those determined against 2.1% on the basis of the cDNA sequence).

Assays of purified human and rabbit LTPs carried out simultaneously under identical conditions gave similar specific

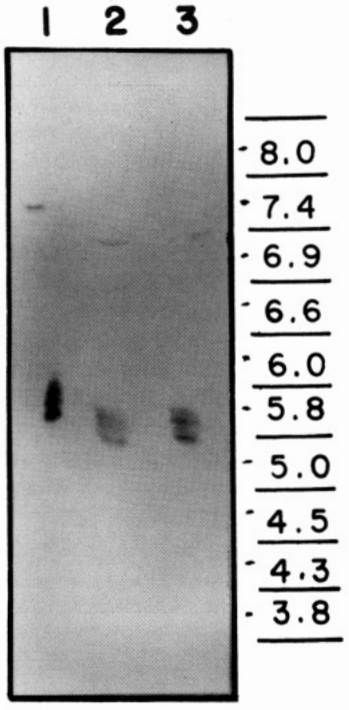

FIGURE 5: Isoelectric focusing of rabbit LTP. Lane 1, 1.2 μg of purified LTP in native sample buffer; lanes 2 and 3, 1.2 μg of purified LTP in 1% mercaptoethanol added to native sample buffer.

activities of CE transfer [3377 nmol h^{-1} (mg of protein)$^{-1}$ for human LTP versus 3781 nmol h^{-1} (mg of protein)$^{-1}$ for rabbit LTP]. Similar specific activities were also found using an alternative assay measuring the transfer of pyrene-labeled CE (Milner et al., 1991); k/L_1[LTP] was 3.37 (min·μM)$^{-1}$ for human LTP and 2.71 (min·μM)$^{-1}$ for rabbit LTP. Using the monoclonal antibody against human LTP, TP2, we were able to inhibit 97% of LTP activity in human plasma by pretreatment with antibody for 4 h at 4 °C based on the assay system used in this study, while only about 43% of the LTP activity in rabbit plasma or pure rabbit LTP could be inhibited in this manner (not shown), agreeing with previous findings (Hesler et al., 1988; Whitlock et al., 1989).

Effect of Lipid on Protein Structure. The behavior of purified rabbit LTP in the presence of lipid microemulsion was investigated by an ultracentifugation binding assay. Pure LTP in the presence of a 6- or 12-fold excess (by PC mass) of microemulsion was recovered predominantly associated with the floated lipid fraction. In the absence of lipid, free LTP partitioned 70:30 between the lower and upper halves of the tube following centrifugation at both protein concentrations studied (Figure 6A). This is an indication of free LTP in the upper half of the tube in the presence of lipid, which should be 3/7 of that in the lower half. In the presence of lipid, LTP is almost entirely in the upper half of the tube, with very little free in the lower half, and correspondingly even less free in the upper half (Figure 6B). The presence of lipid in the sample did not seem to affect subsequent assays for quantitation of LTP, as overall recovery of activity was good and the sum of upper and lower activities was close to the starting/recovered amounts; albumin in the buffer was important for good recovery in the absence of lipid. Recovered lipid was found to be 98% located in the upper half of the tube.

The secondary structure of purified rabbit LTP was estimated by far-UV CD spectroscopy. The spectra were characteristic of a protein containing considerable β structure (Figure 7). Processing of the spectral data by the CONTIN program gave computed fractions of 0.30, 0.39, 0.12, and 0.19 for α-helix, β-sheet, β-turn, and remainder content,

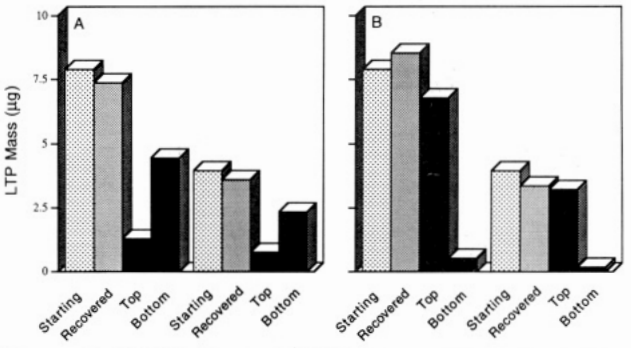

FIGURE 6: LTP binding to the lipid microemulsion. The isolated rabbit LTP was incubated with or without the lipid microemulsion (48 μg as phospholipid) followed by ultracentrifugal separation of the mixture into top and bottom fractions as described under Experimental Procedures. The LTP mass in each fraction was determined by means of the activity assay also described under Experimental Procedures. Panel A shows the results in the absence of the lipid microemulsion: Starting, initial LTP mass (7.9 and 3.95 μg per tube); Recovered, total LTP recovered after incubation; Top and Bottom, LTP distribution between top and bottom fractions, respectively. Panel B shows the results in the presence of the microemulsion also with 7.9 and 3.95 μg of LTP in the same manner.

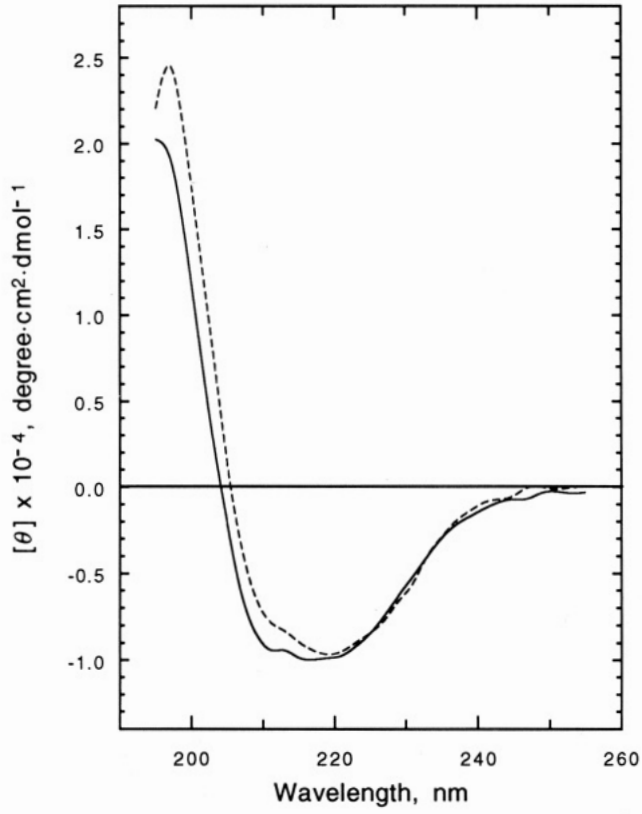

FIGURE 7: Circular dichroism spectroscopy of purified rabbit LTP at 25 °C in 10 mM sodium phosphate (pH 7.4)/150 mM NaCl. Solid line, rabbit LTP alone (72 μg/mL); dashed line, rabbit LTP (72 μg/mL) in the presence of PC/TG microemulsion (504 μg/mL in PC).

respectively, of the protein in buffered 150 mM NaCl. In the presence of TG/PC microemulsion in similar amounts as in the binding studies above (approximately 7-fold excess of microemulsion, by PC mass, over LTP), the corresponding fractional values were 0.26, 0.18, 0.25, and 0.31. The helical content was slightly less upon the presence of lipid, while there was a greater decrease in β-sheet content, with the accuracy of these two parameters being highest for this analytical method (Provencher & Glockner, 1981). Thus, in the presence of lipid, rabbit LTP appears to leave solution and exist predominantly bound to lipid, although this is not

accompanied by much change in the protein helical content but by some disordering of β structure.

DISCUSSION

Rapid and simple purification of rabbit LTP was achieved, and sufficent quantities were obtained to study the behavior of LTP toward lipids and perform protein structural analyses.

For purification, hydrophobic interaction and cation-exchange chromatography relied on the known extreme hydrophobicity (Nagashima et al., 1988) and acidic p*I* of LTP to achieve resolution. The final purification ranged from about 3000 to 5000-fold, which is considerably lower than the values of 28 000–108 000-fold reported in some more recent purifications of human LTP (Jarnagin et al., 1987; Hesler et al., 1987; Kato et al., 1989, Ohnishi et al., 1990). Possibly, the higher content of lipid transfer activity in rabbit plasma (Ha & Barter, 1982) resulted in lower fold purification being sufficient for complete isolation. In one procedure, previously by our lab, in which the results are comparable since the same assay methods were used (Ohnishi et al., 1990), purification was calculated relative to the activity of lipoprotein-depleted plasma, which was acknowledged as probably inaccurately low and would lead to an overestimate of the final purification versus plasma. Somewhat poor recovery of rabbit LTP was obtained by our present procedure (Table I), especially in comparison to that of human LTP by our previous procedure (Ohnishi et al., 1990). This loss of activity is generally seen by us with Butyl-Toyopearl presumably due to the hydrophobic nature of the protein. Our protein appears homogeneous by N-terminal sequencing and amino acid analysis, yet it may have contained a mixture of active and inactive forms, resulting in a lower final specific activity compared to the other laboratories. However, side by side measurements of the final specific activities of rabbit LTP and highly pure human LTP isolated according to our method (Ohnishi et al., 1990) were similar, indicating similar purity resulting from these purification procedures. Our final preparation is extremely stable, in contrast to reports from other labs, and behaved homogeneously in lipid binding assays, in spite of a number of isoforms being detected by isoelectric focusing. Thus, we believe the fold purification we report here to be sufficient to isolate rabbit LTP. In numerous papers where such levels of purification of LTP resulted in apparent partial purity, problems with proteolysis may have been encountered, since this protein is known to be active after proteolysis (Hesler et al., 1989).

Similar to human lipid transfer protein, the rabbit protein migrated in SDS–PAGE at M_r = 74K, much larger than a predicted size of 54 442 from its cDNA (Nagashima et al., 1988). Human LTP is known to be glycosylated (Swenson et al., 1987), and variation in the number of p*I* isoforms (Hesler et al., 1987; Kato et al., 1989) and the elimination of isoforms by neuraminidase (Kato et al., 1989) has been reported. Thus, glycosylation probably also plays a role in generating the isoforms of rabbit LTP detected by isoelectric focusing (Figure 5). By gel permeation chromatography, rabbit LTP behaved as a globular protein of M_r = 83K, similar to results of SDS–PAGE. Human LTP has previously been shown to behave similarly by gel permeation (Hesler et al., 1987; Swenson et al., 1988), whereas native gradient gel electrophoresis has suggested predominantly a dimeric structure (Hesler et al., 1987; Swenson et al., 1989). A misleading result be gel permeation could occur if there was nonspecific interaction with the matrix. Our slightly asymmetrical elution profile tends to support this, and anomalous retention of LTP by a gel filtration column has been reported (Ohnishi et al., 1990). However, previous radiation inactivation studies have suggested an M_r of 70K for rabbit LTP (Ierides et al., 1985). Thus, the question of LTP structure in solution remains unresolve although it is important since self-interactions or interactions with apolipoproteins may be involved in the mechanism or regulation of LTP activity.

Our present structural study of LTP by CD spectroscopy suggested a helical content of about 30%, less than the 40% predicted by Garnier, Osguthorpe, and Robson analysis (Nagashima et al., 1988). However, our CD data were not corrected for the carbohydrate content of the protein. Glycosylation may influence the true conformation of LTP, as well as the spectral data, leading to some discrepancy between results predicted by structural algorithms analyzing primary sequence. The CD spectrum of human LTP was also very similar to that of rabbit (T. Ohnishi and S. Yokoyama, unpublished results). In the presence of lipid microemulsions previously characterized as being 26-nm in diameter (similar to LDL) and consisting of a TG core surrounded by a PC monolayer (Tajima et al., 1983), LTP appeared to bind rapidly and nearly completely to lipid. Changes in the CD spectra upon binding of LTP to lipid were not drastic, with a slight decrease in the α-helical content and some disordering of β-structure occurring. Thus, lipid binding by LTP may not involve amphiphilic helices as for the dissociable apolipoproteins, as helicity did not increase upon binding; furthermore, LTP has been predicted to contain only one short region (residues 264–276 of human LTP, and highly conserved in rabbit LTP) of amphiphilic helix (Drayna et al., 1987; Nagashima et al., 1988).

LTP and apolipoprotein B seem to share common features of extreme hydrophobicity, involvement in neutral lipid binding, and significant predicted β-structure content although LTP dissociates from the lipoproteins while apoB does not. The more pronounced changes in the β structure of LTP upon lipid binding reported here may support this notion that lipid–LTP interaction involves hydrophobic β structures as has been suggested for apolipoprotein B (Yang & Pownall, 1992). It is also known that while intact apolipoprotein B is insoluble and appears anchored to lipoproteins, truncated segments only associate with lipid above a certain length (Talmud, 1992). These observations may help explain LTP's dissociable nature from lipoproteins in spite of its extreme hydrophobicity.

LTP shares significant structural homology with two proteins: bactericidal permeability increasing protein (BPI) (Gray et al., 1989) and lipopolysaccharide binding protein (LBP) (Schumann et al., 1990). The antibacterial function of BPI has been localized to the more basic NH_2-terminal region while the acidic, hydrophobic COOH terminal is proposed to be involved with membrane interaction (Ooi et al., 1987), and, presumably, the lipopolysaccharide binding domain of LBP is also in the NH_2-terminal region of that protein. Then it is tempting to speculate that LTP may have two functional domains also; one for association to lipid particle surfaces and one for lipid binding or transfer. These hypothetical domains may even interact during the function of LTP. There is now evidence that the COOH terminal of LTP contains sequences important for transfer activity (Au-young et al., 1992; Wang et al., 1992) and binding to lipoproteins (Au-young et al., 1992). Antibody to the COOH terminal abolishes transfer activity and carrying of lipid, while leading to a paradoxical increase in binding to lipid particles (Swenson et al., 1989). None of these observations excludes the possibility that lipid binding and transfer may be mediated by different parts of the LTP protein molecule and that one site can influence the other. Perhaps antibody blocks the

lipid carrying site, leading to conformational change in the lipid surface binding site, inducing lipid binding.

Although the preceding discussion is highly speculative, it raises important questions and highlights possible avenues of research which might make progress in the study of LTP. In vitro site-directed mutagenesis studies have begun to provide information on structural features of LTP important for its function (Wang et al., 1991, 1992, 1993; Au-young et al., 1992). The question of whether transfer proceeds via carrier or ternary complex remains, and while a carrier capacity for LTP has been clearly demonstrated (Swenson et al., 1988), the importance of binding for transfer has lead others to favor a ternary mechanism (Au-young et al., 1992). The paradoxical result remains, however, where monoclonal antibody inhibition is accompanied by increased binding to lipid particles (Swenson et al., 1989). To settle such issues, more effort is needed in defining all the functional aspects of LTP, including the active site for transfer as well as possible domains involved in lipid interaction and dimerization sites. The results presented here suggest modes of lipid surface interaction involving β structure and should stimulate further investigation of the structures involved in lipid association by LTP. The preliminary lipid binding results presented here indicate that kinetic constants should be derivable by adapting established protocols (Tajima et al., 1983), and studies coupling lipid binding and kinetics may allow us to distinguish the major mode of transfer employed by LTP as well as to further explore regulatory aspects such as the influence of lipid particle composition or apolipoproteins (Milner et al., 1990; Ohnishi & Yokoyama, 1993) on reaction rate in future studies.

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