Properties of Human Plasma Lipid Transfer Protein in Aqueous Solution and at Interfaces†

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ABSTRACT: Human plasma lipid transfer protein (cholesteryl ester transfer protein) has been characterized for its solution and surface properties. The protein is monomeric in aqueous solution up to 0.62 g/L (11.7 μM) as demonstrated by sedimentation equilibrium. It binds to the surface of a lipid microemulsion having an average diameter of 26 nm made from triolein and egg yolk phosphatidylcholine, with an estimated dissociation constant 1.2×10^{-8} M, and the maximum saturation binding level is 8 protein molecules per particle regardless of the presence of apolipoprotein A-I. Circular dichroism measurement indicated that the protein in solution is predominantly in the β-sheet/β-turn conformation with some α-helix, and this profile does not undergo drastic change by its binding to the lipid surface. The analysis of the behavior of the protein in its monomolecular layer at the air–buffer interface indicated that it is also monomeric at the interface. LTP molecules occupied the same area per amino acid as other apolipoproteins in the monolayer but had a higher collapse pressure of its monolayer (18 dyn/cm), and the protein stayed at the interface even after the overcompressing monolayer far beyond the collapsing pressure up to 40 dyn/cm.

Plasma lipid transfer protein (LTP;[1] cholesteryl ester transfer protein, CETP) catalyzes the transfer of lipids between lipoproteins in blood plasma of certain species of vertebrates (Morton & Zilversmit, 1982; Rueckert & Schmidt, 1990; Tall, 1993). The reaction seems to regulate the distribution of nonpolar lipids, mostly cholesteryl ester and triglyceride, among lipoprotein cores and eventually leads to accumulation of cholesteryl ester in low-density lipoprotein (LDL). Since most of the cholesteryl esters are generated on high-density lipoprotein (HDL) by esterification of cholesterol and because triglyceride is hydrolyzed mainly on very-low-density lipoproteins (VLDL), the LTP reaction causes the transport of cholesteryl ester from HDL to VLDL–LDL as an overall consequence, being accompanied with a countertransport of triglyceride (Morton & Zilversmit, 1983). This proposed function is consistent with lipoprotein profiles in the plasma of animals which are lacking in LTP activity (Oschry & Eisenberg, 1982) and of patients who have a genetic defect of LTP (Inazu *et al.*, 1990; Yamashita *et al.*, 1990).

Human LTP is a glycosylated single-chain polypeptide composed of 476 amino acid residues with a peptide molecular weight of 53 108 (Drayna *et al.*, 1987) and exhibits apparent molecular weights of 66 000 and 69 000 in plasma based on the electrophoretic mobility (Ohnishi *et al.*, 1990), presumably because of the variable extent of the glycosylation (Kato *et al.*, 1989; Stevenson *et al.*, 1993). LTP has been shown to interact with lipid on the membrane surface, lipoproteins, and substrate lipid molecules, and it has also been shown to be extremely hydrophobic in nature among water-soluble proteins (Pattnaik & Zilversmit, 1979; Morton, 1985). It seems to have a higher affinity for HDL than other lipoproteins in plasma as demonstrated by electrophoresis, and this affinity is also modified by some species specificity (Marcel *et al.*, 1990; Hayek *et al.*, 1992). However, the majority of the activity is found in the lipoprotein-free fraction of plasma after precipitation or ultracentrifugal floatation of lipoproteins (Groener *et al.*, 1984). These facts have resulted in some controversy about the hydrophobic nature of the protein and its reaction mechanism as to whether it is a shuttle of substrate lipids between lipoproteins (Barter & Jones, 1980; Swenson *et al.*, 1988) or forms a ternary transfer complex (Ihm *et al.*, 1982). Moreover, very little information is available about the structure of LTP in an aqueous solution and on the lipid membrane surface in relation to its reaction mechanism or its active form.

In order to understand further the behavior of LTP in plasma and its reaction mechanism, it is important to know how the protein behaves in aqueous solution and how it interacts with the lipid and the lipoprotein surface. Thus, physicochemical properties of LTP in solution and at interfaces are essential, and the securing of this basic information is the subject of this paper.

EXPERIMENTAL PROCEDURES

Chemicals. Egg yolk phosphatidylcholine was purchased from Avanti Polar-Lipids, Inc., and triolein and standard proteins were from Sigma. Other chemicals used in the present study were of the highest quality commercially available.

Isolation of LTP and Apolipoprotein A-I from Human Plasma. Human LTP was isolated according to the method previously reported (Ohnishi *et al.*, 1990). The final preparation contained duplex bands in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of 66 and

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[1] Abbreviations: apo, apolipoprotein; C, circular dichroism; CETP, cholesteryl ester transfer protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LTP, lipid transfer protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VLDL, very-low-density lipoprotein.

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69 kDa. Both bands had the same N-terminal amino acid sequence and the same specific activity for cholesteryl ester transfer, and the 69-kDa band was predominant (95%) in the particular preparation used for the present experiments. The final preparation was concentrated by a small butyl-Toyopearl column (10 mL) using the same condition for isolation of LTP except for the one-step elution with water and stored at −75 °C as 1.4 mg/mL in 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM sodium chloride and 0.1 mM EDTA. All the experiments in the present paper were performed in the same buffer unless the experimental condition was specified. Apolipoprotein (apo) A-I was isolated from human HDL fraction and dissolved in the buffer as previously described (Yokoyama *et al.*, 1982).

Determination of the Protein Concentration. Concentration of apo A-I was determined from a specific extinction coefficient [1.046 cm^{-1} $(mg/mL)^{-1}$ at 280 nm for peptide moiety] (Tajima *et al.*, 1983). The concentration of LTP was routinely measured by a Pierce BCA protein assay kit using bovine serum albumin as a standard, which was highly consistent with the value confirmed by amino acid analysis using a Beckman 6300 amino acid analyzer within 1% error. The extinction coefficient of LTP was determined experimentally to be 0.85 cm^{-1} $(mg/mL)^{-1}$ at 278.4 nm on the basis of the protein concentration measured by amino acid analysis, which agreed with the value of 0.78 cm^{-1} $(mg/mL)^{-1}$ at 280 nm calculated from its amino acid composition deduced from cDNA sequence (Drayna *et al.*, 1987).

Preparation of Lipid Microemulsion. Lipid microemulsions were prepared according to the method previously described from triolein and egg yolk phosphatidylcholine in a homogeneous diameter of 26 nm that is roughly the same size as LDL (Tajima *et al.*, 1983; Ohnishi & Yokoyama, 1993). The two lipids in a weight ratio of 1:1 were sonicated in the buffer. The emulsions in a homogeneous size were isolated by ultracentrifugation and gel permeation chromatography and stored under argon at 4 °C. The weight ratio of triglyceride to phospholipid was 1.2 in the final preparation used.

Gel Permeation Chromatography. Fifty micrograms of LTP was analyzed on a Superose 12 column (10/30) connected to an FPLC system (Pharmacia) at 4 °C. The column was equilibrated with 10 mM phosphate buffer with 0.1 mM EDTA in the presence and absence of 0.15 M NaCl at a flow rate of 0.5 mL/min, and the protein was monitored by the absorbance at 254 nm (0.1 AUFS). To calibrate the column, mouse immunoglobulin G (160 kDa, 40 μg), bovine serum albumin (66 kDa, 120 μg), bovine β-lactoglobulin A (35 kDa, 40 μg), horse myoglobin (17 kDa, 15 μg), and bovine cytochrome *c* (12.4 kDa, 15 μg) were used as molecular weight standards.

Analytical Ultracentrifugation. LTP solution in the phosphate buffer was analyzed in a Beckman Model E analytical ultracentrifuge equipped with an electronic speed control system, photoelectric scanner, and an RTIC temperature control system. The sample solution, 100 μL, was loaded into a 12-mm double-sector charcoal-filled Epon cell equipped with sapphire windows. Sedimentation equilibrium runs were performed at 20 °C at 10 000 rpm for 48 h before equilibration traces were taken. Molecular weight of the peptide was calculated from the slope of the plot of logarithm of protein concentration (ultraviolet absorbance at 278 nm) against the square of the distance of the position from the center of rotation, as M_r = (the slope) × $2RT/\{(1 - \nu r)\omega^2\}$, where R is the gas constant, ν is partial specific volume of the protein, r is density of the solvent, and ω is the angular velocity of the rotor. The value of ν was calculated for LTP as 0.706 assuming that 23% of the molecule is the carbohydrate moiety [(69000 − 53108)/69000] and the average partial specific volume of carbohydrate moiety is 0.65 (Gibbons, 1966).

Circular Dichroism (CD) Spectroscopy. The CD spectra were taken in a Jasco J-720 spectropolarimeter equipped with a thermostatic cell holder at 25 °C. The instrument was calibrated with ammonium *d*-(+)-10-camphorsulfonate at 290.5 and 192 nm, and with *d*-(−)-pantoyllactone at 219 nm. LTP (107.3 μg/mL) in the buffer solution was placed in a 0.05-cm cell in the absence and presence of the lipid microemulsion (536.4 μg of phosphatidylcholine/mL). Ten scans were performed, and the signal due to the background solvent was subtracted. The spectrum of the molar residual ellipticity was analyzed by a computer program (CONTIN version 1.0) for prediction of secondary structure (Provencher & Glöckner, 1981). No correction for carbohydrate moiety was included in the CD analysis.

Equilibrium Binding of LTP to Lipid Microemulsion. After preincubation of lipid microemulsion (10 μg of phosphatidylcholine) in the presence and absence of human apo A-I (4 μg) at room temperature for 10 min, various amounts of LTP were mixed with the emulsion in the buffer, 200 μL, containing 0.5% bovine serum albumin and incubated at a room temperature for 60 min. The mixture was centrifuged in a Beckman TL100 table-top ultracentrifuge at 25 °C for 30 min using a TL100.1 rotor at 99 000 rpm. Each top and bottom 100-μL fraction was obtained by removing the top fraction manually using a Hamilton's syringe with a flat-tipped needle. The amount of LTP was measured in each fraction by the transfer activity assay between HDL and LDL using radioisotope-labeled cholesteryl ester according to the method described elsewhere (Ohnishi *et al.*, 1990). The activity of LTP measured by this method was not affected by the presence of microemulsion (at most 10 μg of phospholipid) or apo A-I (at most 4 μg) because of the use of excess amonts of donor and acceptor lipoproteins (approximately 230 μg of phospholipid and 335 μg of total apolipoproteins) (Ko *et al.*, 1993). The background free LTP in the top fraction was calculated from the amount of LTP in each bottom fraction and a partitioning factor of free LTP between the top and bottom fractions measured in the control samples without the lipid emulsion. As lipid-free LTP slowly moved to the bottom fraction at least over the initial 30 min of centrifugation at 25 °C (2.5%/min), lipid-bound LTP in the top fraction also decreased slowly (1.9%/min). Therefore, the bound and free LTP in the top fraction were assumed to be nearly in equilibrium. Binding constants (dissociation constant and maximum binding level) for the equilibrium binding were thus calculated from the concentration of free and bound LTP and of lipid microemulsion in the top 100-μL fractions (Ko *et al.*, 1993).

Monomolecular Layer of LTP at the Interface of the Buffer and Air. The surface pressure of the LTP monomolecular layer was measured on the surface of the buffer in a mgw Lauda Langmuir Filmwaage equipped with an IEEE-computer control. Water used in the monolayer experiment was distilled in an all-glass apparatus. The buffer was foamed with air for 10 min, and the surface layer was removed by vacuum-sucking before use in order to remove surfactant contamination. Every glass apparatus was washed with enzyme-free soap, treated with nitric acid, and rinsed thoroughly with glass-distilled water. The samples were applied onto the buffer surface using a Hamilton's syringe via a slide glass partially immersed in the buffer from the surface.

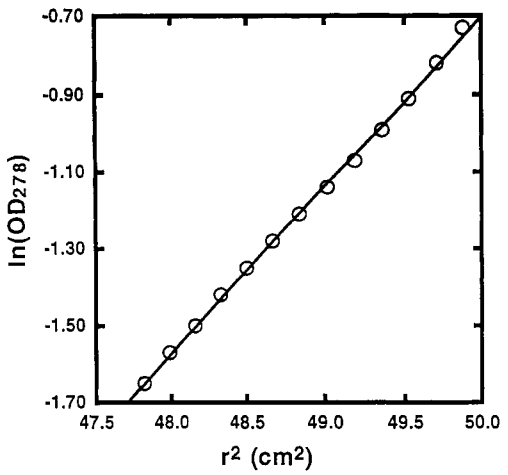

FIGURE 1: Sedimentation equilibrium study of human LTP. LTP (120 μL, 8.8 μM) in 10 mM phosphate buffer with 150 mM NaCl and 0.1 mM EDTA was ultracentrifuged at 20 °C at 10 000 rpm for 48 h, and the distribution of the protein concentration in the cell was measured spectrophotometrically at 278 nm. The logarithm of the absorbance was plotted against the square of the distance from the rotation center. The slope of the plot was calculated using a least-squares linear regression method.

The monolayer was compressed and expanded repeatedly between 960 and 200 cm^2 with rates of 7.5–45 cm^2/min. Surface pressure (π, dyn/cm) was recorded as a function of the area of the monolayer (A). A portion of the data of very low surface pressure ($\pi \leq 1.0$ dyn/cm) was analyzed according to the ideal gas equation, $\pi(A - A_0) = nRT$, using a linearized plot of $\pi A = \pi A_0 + nRT$. The value of n (number of protein molecules) was obtained from the y intercept of the πA against π plot, and the apparent molecular weight of the protein in the monolayer was calculated from the weight of the protein in the monolayer (Adamson, 1976; Yokoyama *et al.*, 1985). From the slope of the plot, the area occupied by the protein molecule, A_0, was calculated in this gaseous phase. The curve with higher surface pressure was analyzed to determine the collapsing pressure of the monolayer as the pressure where the second derivative of $\pi = f(A)$ becomes positive during the compression, and to calculate some empirical parameters according to the equation, $B = \pi\{A - A_{00}(1 - k\pi)\}$, where B, A_{00}, and k are empirical constants (Shen & Scanu, 1980; Yokoyama *et al.*, 1985).

Other Analytical Methods. Concentrations of lipids were determined using enzymatic assay kits for triglyceride and choline-containing lipid purchased from Wako Pure Chemical (Richmond, VA). Spectrophotometric measurement was carried out on a Hitachi U-2000 spectrophotometer.

RESULTS

The solution of LTP was analyzed by gel permeation chromatography on a Superose 12 column (Pharmacia) equilibrated with 10 mM phosphate buffer (pH 7.4) with 0.1 mM EDTA in the presence and absence of 0.15 M NaCl. In the presence of NaCl, the elution volume of the standard proteins, immunoglobulin, bovine serum albumin, β-lactoglobulin, myoglobin, and cytochrome *c*, constructed a reasonable calibration of the column for their molecular weight. However, LTP was eluted behind cytochrome *c*, giving an estimated apparent molecular mass less than 10 kDa. When the chromatography was performed without NaCl, the elution volume of LTP was between those of β-lactoglobulin and myoglobin, giving an estimated apparent molecular mass of 31 kDa. Thus, LTP was eluted much later than its monomeric molecular mass position, showing that this method is not suitable for the analysis of the behavior of this protein in solution. The data were consistent with its behavior in many other conventional gel permeation chromatographies (Ohnishi *et al.*, 1990).

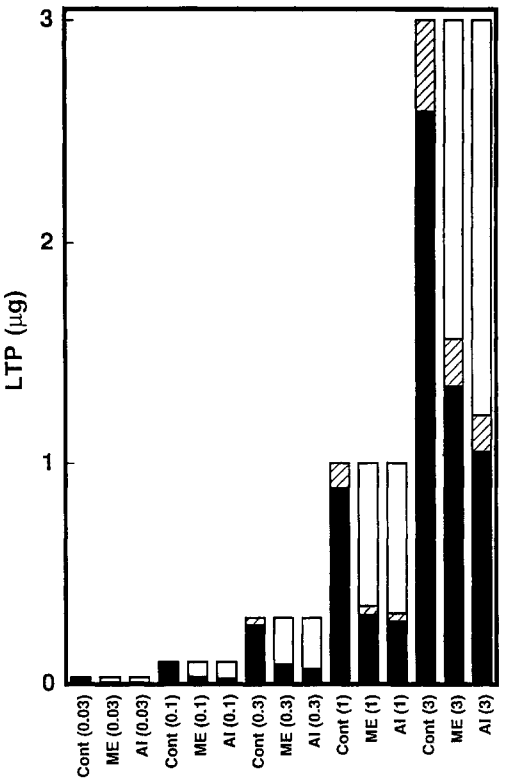

FIGURE 2: Equilibrium binding of human LTP to the lipid microemulsion. LTP (0.03, 0.1, 0.3, 1, or 3 μg), as indicated by the numbers in parentheses, was incubated with the microemulsion only (10 μg of phosphatidylcholine) (ME) and with the emulsion preincubated with human apo A-I (4 μg) (AI) in the phosphate buffer containing 0.5% bovine serum albumin at room temperature for 60 min. Control was with neither emulsion nor apo A-I (Cont). The mixture was ultracentrifuged at 25 °C at 99 000 rpm for 30 min, and the amount of LTP in each top and bottom half fraction was determined on the basis of its cholesteryl ester transfer activity. The amount of free and bound LTP in the top fraction was calculated from the distribution of free LTP between the top and bottom fraction without the microemulsion (see Experimental Procedures). The closed portion of the bars shows the amount of LTP in the bottom fraction, the hatched portion shows that of free LTP in the top fraction, and the open portion of the bars shows that of lipid-bound LTP in the top fraction.

Figure 1 demonstrates a typical result of a sedimentation equilibrium experiment on a LTP solution. The plot of logarithm of the protein concentration (ultraviolet absorbance at 278 nm) against the square of the distance from the rotation center gave a good straight line within a concentration range between 0.23 and 0.62 g/L (4.3 and 11.7 μM) as peptide moiety. From the slope of this plot, the apparent molecular weight of LTP was calculated to be 66 954 ± 613 including the carbohydrate moiety. This value is in very good agreement of its estimated molecular weight (69 000) on SDS–PAGE. Thus, LTP is monomeric in an aqueous solution even at very high concentrations, at least in neutral pH and at physiological ionic strength.

The binding of LTP to the lipid microemulsion surface was studied. LTP bound to the lipid microemulsion in the absence and presence of human apo A-I even when apo A-I was sufficient to presaturate the surface of the microemulsion. Figure 2 shows the distribution of LTP between the top (open and hatched bars) and the bottom (closed bars) fractions in

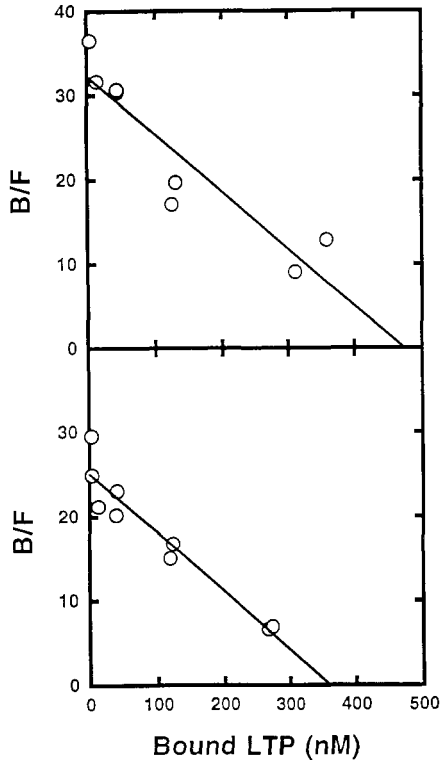

FIGURE 3: Scatchard plot of binding data of LTP to the microemulsion in the absence (top) and presence (bottom) of apo A-I. The experimental condition and the calculation of the amount of free and bound LTP was described in Experimental Procedures and the legend for Figure 3. The ratio of the amount of bound to free LTP was plotted against the concentration of bound LTP. The plot was fitted with a least-squares linear regression method.

ultracentrifuge tubes. The amount of LTP in each fraction was measured as its activity calibrated on the basis of the specific activity of LTP. The amount of free LTP (hatched bars) in the top fraction containing the emulsion was calculated from the amount of LTP in the bottom fraction and its partitioning factor between the top and bottom fractions without emulsion. The bound LTP (open bars) was determined by subtraction of the free LTP from the total LTP in the top fraction. Since centrifugation was performed at 25 °C for 30 min, the binding of LTP was assumed to be in equilibrium within the top fraction. The data were analyzed by Scatchard plot according to an equation for equilibrium binding, $[LTP_b]/[LTP_f] = K(NP - [LTP_b])$, where $[LTP_b]$ and $[LTP_f]$ are the concentrations of the bound and free forms of LTP, respectively, P is the concentration of the lipid microemulsion, N is the maximum binding of LTP per microemulsion, and K is an association constant, all in the top fraction. Since LTP had been shown to be monomeric in an aqueous phase, the equation should directly represent equilibrium between a single monomeric state of LTP in solution and the bound form to the lipid surface. The ratio of the concentration of bound LTP to free LTP was plotted against the concentration of bound LTP in Figure 3. According to the above equation, the dissociation constant (K_d) will be the inverse of the slope, and the maximum binding of LTP (N) is calculated from the x intercept of the plot. The K_d values were 12.4 nM and 13.2 nM, and the N values were 1.52 and 1.32 amino acid/phospholipid (mol/mol) in the absence and presence of apo A-I, respectively. Thus, the presence of apo A-I did not have a significant effect on the binding profile of LTP even though apo A-I supposedly presaturated the surface of the microemulsion prior to the LTP binding. Table 1 summarizes the binding parameters of LTP in comparison to those of

Table 1: Parameters for Binding of LTP and Apo A-I, A-II, C-II, C-III, and E to the Lipid Microemulsion

	K_d (nM)	N^a molecules/particle	N^a amino acid/PC (mol/mol)
LTP without apo A-I	12.4 ± 2.2	8.3 ± 1.1	1.52 ± 0.21
LTP with apo A-I	13.2 ± 1.5	6.7 ± 0.7	1.23 ± 0.12
apo A-I[b]	160 ± 70	5.6 ± 1.0	0.58 ± 0.09
apo A-II[b]	250	24	1.44
apo C-II[b]	450 ± 27	31.0 ± 5.6	0.93 ± 0.17
apo C-III[b]	530 ± 13	31.6 ± 6.6	0.96 ± 0.20
apo E[c]	1180 ± 100	6.9 ± 1.3	0.80 ± 0.15

[a] The maximum binding of the protein to the microemulsion. The values for molecules per particle were calculated assuming 2600 phosphatidylcholines per particle. [b] The values are from a previous report (Tajima *et al.*, 1983). [c] The values are from a previous report (Yokoyama *et al.*, 1985).

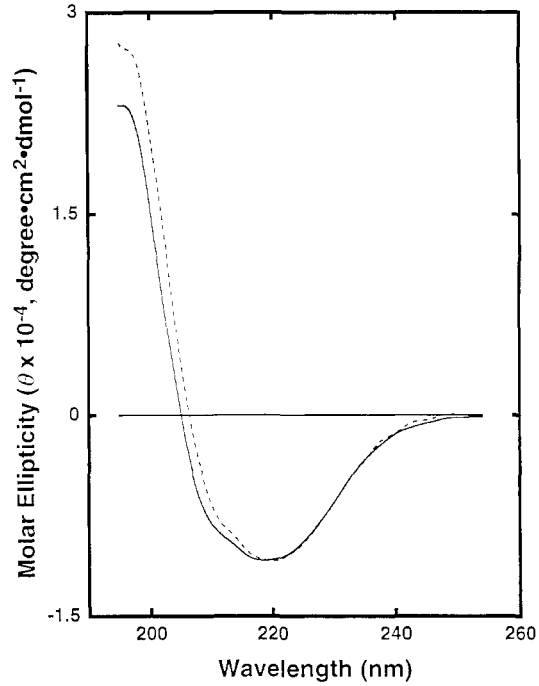

FIGURE 4: Circular dichroism spectroscopy of human LTP. The solid line shows LTP (107.3 μg/mL) at 25 °C in 10 mM phosphate buffer with 150 mM NaCl and 0.1 mM EDTA, and the dashed line shows LTP in the presence of the lipid microemulsion (536.4 μg of phosphatidylcholine/mL). Ninety-eight percent of LTP is lipid-bound form in the latter condition according to the binding parameters listed in Table 1. The molar ellipticity was calculated on the basis of the mean amino acid residue weight derived from the amino acid sequence of LTP.

representative apolipoproteins measured in our previous publications (Tajima *et al.*, 1983; Yokoyama *et al.*, 1985). The N values were also calculated as the number of LTP molecules per particle assuming that each emulsion particle contains 2600 phosphatidylcholine molecules. The dissociation constants of LTP, on the order of 10^{-8} M, are significantly lower than those for most apolipoproteins (A-I, A-II, C-II, C-III, and E) measured under similar conditions. On the other hand, the maximum binding levels (N values), 1.52 and 1.32 amino acid/phospholipid (mol/mol) or 8.3 and 6.7 proteins per microemulsion in the absence or presence of apo A-I, respectively, are not substantially different from those of the apolipoproteins.

The CD spectrum of LTP was measured for its aqueous solution and also for its lipid-bound stage (Figure 4). The profile of the spectrum is very similar to that of rabbit LTP (Ko *et al.*, 1993). The Provencher–Glöckner analysis

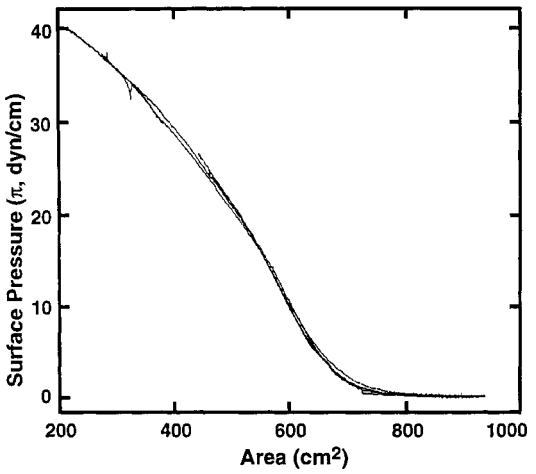

FIGURE 5: Monomolecular layer of LTP at the interface of the buffer and air. LTP (56 μg) was applied to the surface of the phosphate buffer in a Lauda Langmuir Filmwaage as described in Experimental Procedures. The monolayer was compressed and expanded 4 times between 960 and 200 cm² (0.06 and 0.28 μg of protein/cm²) with rates of 7.5–45 cm²/min. Surface pressure (dyn/cm) was recorded as a function of the area of the monolayer.

(Provencher & Glöckner, 1981) of the profile gave conformation estimates of 29% α-helix, 38% β-sheet, 10% β-turn, and 23% remainder. This profile did not undergo drastic change even when most of LTP was transformed to the lipid-bound stage by adding the lipid microemulsion. The same analysis gave estimates of 27% α-helix, 37% β-sheet, 16% β-turn, and 21% remainder when 98% of LTP was bound to the lipid microemulsion. Thus, the conformation of LTP does not seem to undergo drastic alteration when it binds to the lipid surface from the aqueous phase. This result was also very similar to that of the CD study of rabbit plasma LTP (Ko *et al.*, 1993).

In order to assess the behavior of the protein at the interface, the force-area curve of the LTP monolayer was analyzed. Figure 5 shows four repeated compressions of the same LTP monolayer with relatively long-time intervals from 20 to 120 min between the experiments. The monolayer showed higher collapsing pressure, some 18 dyn/cm, than these values for apolipoproteins (12–14 dyn/cm) (Shen & Scanu, 1980; Yokoyama *et al.*, 1985). Beyond this point, the surface pressure still went up to as high as 40 cm/dyn as the monolayer was overcompressed, which has never been achieved with apolipoproteins. As well, the curves of the repeated compression were precisely superimposable on each other no matter how long or how short an interval was given between the compressions and even after the compression to very high surface pressure as mentioned above. These data demonstrate that LTP has a very high affinity for the hydrophobic surface, which is consistent with its low dissociation constant for interaction with the lipid surface. When the low-pressure portion of the curve ($\pi \leq 1$ dyn/cm), where the molecular interaction of the protein is presumably minimal, was analyzed using the ideal gas equation (see Experimental Procedures), the linearized plot of the data demonstrated a good straight line and an apparent molecular weight was calculated from its y intercept, nRT (Figure 6). The calculated value for the protein moiety was 45 506 ± 4007 in very good agreement with the value obtained from its amino acid sequence, 53 108. Thus, it is reasonable to assume that LTP is also monomeric at the interface. The area occupied by LTP at the interface is calculated from the slope of the plot, the A_0 value, as 1.12 × 10^{-16} m²/molecule or 23.5 Å²/amino acid. This value is more or less the same as the area per amino acid occupied by α-helical apolipoproteins at the interface (Shen & Scanu, 1980; Yokoyama *et al.*, 1985). Thus, the data are consistent with the maximum binding level of LTP to the lipid microemulsion surface and are also similar to those values found for α-helical apolipoproteins. Analysis of the part of the curve with higher surface pressure ($\pi \leq 10$ dyn/cm) according to the empirical equation (see Experimental Procedures) yielded some parameters to describe the curve (Shen & Scanu, 1980; Yokoyama *et al.*, 1985). Table 2 lists these parameters including absolute collapse pressure in comparison to those obtained for some α-helical apolipoproteins. The molecular area of LTP at the interface is again similar to that of apolipoproteins. A substantially lower k value of LTP may indicate less lateral compressibility of the LTP molecule in the monolayer, or in other words higher rigidity of the molecule than in the case of apolipoproteins.

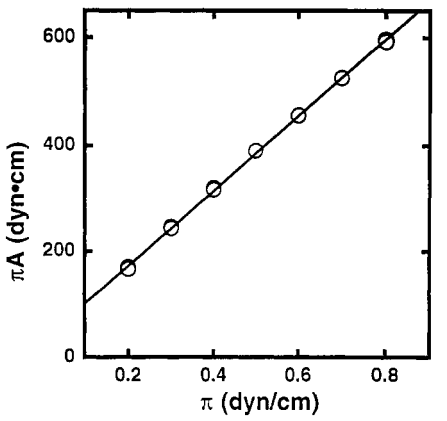

FIGURE 6: Analysis of the monomolecular layer experiment at the initial phase of compression. A portion of the data of the low surface pressure ($\pi \leq 1.0$ dyn/cm) was analyzed according to the ideal gas equation described in Experimental Procedures. The product of the surface pressure and the area of monolayer was plotted against the surface pressure. The slope and the y intercept were calculated by a least-squares linear regression method.

Table 2: Parameters of Protein Monolayers at the Air–Water Interface

	apo E[a]	apo A-I[b]	LTP
collapse pressure (dyn/cm)	14	14	18 (40)
A_0 (Å²/amino acid residue)	24.0	24.0	23.5
molecular weight	30 300 ± 1700	27 000 ± 2500	45 500 ± 4000
A_{00} (Å²/amino acid residue)	20.8	21.6	22.4
$k \times 10^2$ (cm/dyn)	3.0	3.7	1.2
$B \times 10^{-6}$ (erg/g)	2.1	3.8	1.2

[a] The values are from Yokoyama *et al.* (1985). [b] The values are from Shen and Scanu (1980).

DISCUSSION

Human LTP has been characterized for its behavior in aqueous solution, on the surface of the lipid microemulsion, and at the air–buffer interfaces. There are some controversial reports about the functional molecular weight of LTP in aqueous solution. Ierides *et al.* reported that LTP works as a monomer in an aqueous solution in a radiation inactivation study, using partially purified LTP (Ierides *et al.*, 1985). On the other hand, Tall and his colleagues reported that LTP was dimeric in a nondenatured gradient gel electrophoresis system (Swenson *et al.*, 1989; Hesler *et al.*, 1987) or quasielastic light scattering (Takahashi *et al.*, 1993).

LTP was eluted at the position of slightly lower molecular weight than bovine serum albumin form Bio-Gel A-0.5m (Morton & Zilversmit, 1982) and from Sephacryl S-200

(Tollefson *et al.*, 1985) in physiological ionic strength. A significant shift of the eluted position of LTP from Sephadex G-200 columns by the binding of Fab fragment of the monoclonal antibody may also indicate that LTP is monomeric in diluted solution (Swenson *et al.*, 1989). However, our previous observation indicated that the behavior of LTP on gel permeation chromatography may not be reliable for determination of the molecular weight of human LTP because of its hydrophobic nature (Ohnishi *et al.*, 1990). LTP eluted at 1.5 times the total volume of the HW-55 column. The analysis on a TSK G3000SW HPLC column also gave a retarded elution of LTP. Even on ion-exchange chromatography with Toyopearl, Sepharose, and cellulose gels, LTP showed strong interaction with the gel matrix at high ionic strength (Ohnishi *et al.*, 1990). Since a higher ionic strength and more hydrophobic gel matrix tend to cause more retarded elution of LTP, this is perhaps due to the hydrophobic interaction between the gel and the protein. Such interaction seems to have led to largely inconclusive results in gel permeation chromatography experiments. The present result with the Superose 12 column was essentially identical to our previous results with a TSK G3000SW column (Ohnishi *et al.*, 1990). LTP eluted behind cytochrome *c* because of hydrophobic interaction between LTP and the gel at physiological ionic strength, and such ionic interaction was not completely eliminated even at low ionic strength. Thus gel permeation chromatography may not be a convincing method to determine the active molecular weight of LTP and thereby may have misled the conclusion of an earlier report (Busch *et al.*, 1987).

We should also note the difference in hydrophobic nature between human and rabbit LTP. HW-55 column chromatography was invoked as the final step in the purification of human LTP, utilizing its strong interaction with this particular gel. However, it was not effective for purification of rabbit LTP. Superose column chromatography gave a reasonable M_r of 83K for rabbit LTP (Ko *et al.*, 1993). Therefore, the apparent hydrophobicity of rabbit LTP in solution seems to be less than that of human LTP in spite of the high similarity of their primary sequence.

On the other hand, the result of the sedimentation equilibrium study was conclusive that the protein is monomeric in solution up to very high concentration under physiological ionic strength and pH. Since the activity of LTP is linearly proportional to its concentration even in much lower concentration (Ohnishi *et al.*, 1990; Ohnishi & Yokoyama, 1993), it is reasonable to assume that this monomeric LTP is an active form.

LTP binds to the lipid surface with higher affinity than that of apolipoproteins previously measured (Tajima *et al.*, 1983; Yokoyama *et al.*, 1985), and this affinity is not affected by the presence of apo A-I on the lipid surface. Thus, at least on the spherical microemulsion with the same size as LDL, apo A-I neither enhances nor inhibits the physical interaction of LTP with the lipid surface in spite of the fact that apolipoproteins including apo A-I are an almost absolute requirement for the activation of LTP in the reaction between the microemulsions (Nishikawa *et al.*, 1988; Milner *et al.*, 1991; Ohnishi & Yokoyama, 1993). One unresolved question relates to whether or not LTP competes with apolipoproteins for the binding site of the lipid emulsion surface. The high affinity of LTP for the microemulsion surface may explain the absence of a difference in the binding parameters, with and without apo A-I, because apo A-I may easily be displaced by LTP. The binding parameters of LTP may be verified by an immunochemical LTP mass assay which is to be set up using the monoclonal antibody raised against rabbit LTP cross-reacting with human LTP in our laboratory (Ko and Yokoyama, unpublished data). It should also be noted that moderate affinity of LTP for HDL found in plasma (Marcel *et al.*, 1990; Hayek *et al.*, 1992) may not be a complete analogy of the LTP–lipid association demonstrated in this paper due to the significant difference in the structure of lipid–protein particles.

The question of the reaction mechanism still remains to be answered. A low dissociation constant of LTP for the lipid surface does not necessarily mean that LTP binds tightly to the surface. It still may dissociate from the lipid particle if the absolute on/off rate of the interaction may be very rapid, and carry lipids between the particles in a carrier mechanism in spite of the fact that most of the protein is in lipid-bound form. Therefore, the high affinity of LTP for the lipid surface may not directly suggest a ternary complex model for the reaction mechanism. It is also possible that the lipid-bound LTP may not be an active form by itself for the transfer reaction because an activator apolipoprotein did not have an effect on the binding parameters of LTP.

The overall conformation of LTP does not undergo drastic change when the protein binds from the aqueous phase to the lipid surface. However, the CD result does not exclude the possibility that the protein may undergo major conformational reorganization, maintaining its structural integration, such as the opening of a hydrophobic surface that may be masked in aqueous solution by a hinge-like domain (Breiter *et al.*, 1991).

The analysis of the monolayer of LTP indicated that it is likely to behave as a monomer at the interface as well as in aqueous solution. The area occupied by LTP at the interface is similar to that occupied by most apolipoproteins. This was also consistently demonstrated by the saturation level of the lipid surface by the bound LTP. The most striking nature of LTP at the interface is the extremely high surface pressure of the monolayer when it is overcompressed and the fact that protein stays at the interface even after repeated compression far beyond the collapsing pressure. Thus, LTP has been shown to be an exceptionally surface-active protein, perhaps the most surface-active protein ever reported among water-soluble proteins.

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