Isolation and Partial Characterization of the Inactive and Active Forms of Human Plasma Phospholipid Transfer Protein (PLTP)*

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From the ‡Department of Molecular Medicine, National Public Health Institute, Biomedicum, P. O. Box 104, Helsinki FIN-00251, Finland and §Research Division, Research and Development Center, BML Inc., 1361-1 Matoba, Kawagoe, Saitama 350-1101, Japan

Plasma phospholipid transfer protein (PLTP) plays an important role in lipoprotein metabolism. Two forms of PLTP exist in human plasma, one catalytically active (high activity form, HA-PLTP) and the other inactive (low activity form, LA-PLTP) (Oka, T., Kujiraoka, T., Ito, M., Egashira, T., Takahashi, S., Nanjee, N. M., Miller, N. E., Metso, J., Olkkonen, V. M., Ehnholm, C., Jauhiainen, M., and Hattori, H. (2000) J. Lipid Res. 41, 1651–1657). The two forms are associated with macromolecular complexes of different size. The apparent size of LA-PLTP is 520 kDa and that of HA-PLTP is 160 kDa. Of the circulating PLTP mass only a minor portion is in the HA-PLTP form in normolipidemic subjects. In the present study we have isolated and partially characterized the LA and HA forms of PLTP. Both LA- and HA-PLTP bind to heparin-Sepharose and can be separated by elution with 0–0.5 M NaCl gradient, with HA-PLTP displaying higher affinity for the matrix. LA-PLTP was further purified using hydrophobic butyl-Sepharose and anti-PLTP immunoaffinity chromatography steps. HA-PLTP was subjected to a second heparin-Sepharose step and hydroxylapatite chromatography. Analysis of the two forms of PLTP by SDS-PAGE, Western blotting, immunoprecipitation, and gel filtration demonstrates that LA-PLTP is complexed with apoA-I whereas HA-PLTP is not. Instead, HA-PLTP copurified with apoE. Based on these findings we suggest a model in which nascent PLTP enters the circulation as a high specific activity form not associated with apoA-I. During or after the transfer of lipolytic surface remnants to HDL, PLTP is transferred to apoA-I-containing HDL particles and thereby becomes part of the low activity complex.

Both epidemiological and clinical studies provide strong evidence that low levels of high density lipoproteins (HDL) is a major risk factor for the development of coronary heart disease (1–5). The ability of HDL to protect against atherosclerotic coronary artery disease is well documented, and although the exact molecular mechanism(s) behind this finding is still unsolved, it is thought to be due to the role of HDL in reverse cholesterol transport (6). The HDL in human plasma consist of several subpopulations of particles of distinct structure, function, and composition. This heterogeneity, which is the result of continuous remodeling of HDL by plasma factors, has important implications in terms of the cardioprotective functions of HDL (7).

Plasma phospholipid transfer protein (PLTP) plays an essential role in the metabolism of HDL. Its role in the transfer of surface remnants from triglyceride-rich particles, very low density lipoproteins, and chylomicrons, to HDL during lipolysis is of importance for the maintenance of HDL levels (8–10). It also modulates the size and composition of HDL particles (11, 12), a function important for the reverse cholesterol transport process. We recently reported the presence of two forms of PLTP in plasma (13), one catalytically active and the other inactive. Size-exclusion chromatography demonstrates that these two forms are associated with macromolecular complexes of different size: the active PLTP elutes in the position of large HDL particles, and the inactive PLTP elutes between HDL and low density lipoprotein.

To gain insight into the regulation of the different forms of PLTP in the context of lipoprotein metabolism and to elucidate the mechanisms involved in the generation of these two PLTP populations we decided to isolate and characterize the active and inactive forms of plasma PLTP. We now report that the inactive and active forms of PLTP can be separated using heparin-Sepharose affinity chromatography and that they can be further purified by hydrophobic chromatography and anti-PLTP immunoaffinity chromatography. Using immunoprecipitation with anti-PLTP and anti-apoA-I antibodies we demonstrate that the inactive form of PLTP is complexed with apoA-I whereas the active form copurifies with apoE.

MATERIALS AND METHODS

Collection of Human Plasma—Normolipidemic human plasma was obtained by plasmapheresis. The protease inhibitors Trasylol, 50 units/ml; dichloroisocoumarin (DCIC), 0.4 mM; E-64, 1 mM; 3-amidinophenylmethanesulfonyl fluoride (APMSF), 1 mM, and leupeptin, 10 μM, were immediately added to the plasma.

Assay of PLTP Activity—PLTP activity was measured using the radiometric assay described by Damen et al. (14) with minor modifications (11).

Analysis of Human PLTP Mass—Human PLTP mass was measured using the PLTP enzyme-linked immunosorbent assay method (15, 16).

Plasma Lipid and Lipoprotein Analysis—Total serum cholesterol (Roche Diagnostics GmbH, Mannheim, Germany, catalog no. 1489232), serum triglycerides (Roche Diagnostics GmbH, Mannheim, Germany, catalog no. 1489232).
catalog no. 1488872), phospholipids (WAKO Chemicals GmbH, Neuss, Germany, phospholipids B, code 990–54009), and free cholesterol (WAKO Chemicals GmbH, Neuss, Germany, code 274-47109) were measured using commercial kits. Cholesterol esters were calculated by subtracting the free cholesterol from the total cholesterol value.

Lipoproteins were obtained by size-exclusion chromatography using two HR 10/30 Superose 6 (Amersham Biosciences) gel filtration columns in tandem. Elution of lipoproteins was carried out using 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 1 mM EDTA, pH 7.4 (PBS). Elution was performed at room temperature with a flow rate of 0.25 ml/min, and 0.5-ml fractions were collected.

Preparation of antibodies—The monoclonal antibody JH66 against PLTP was produced and isolated as previously described (15). As judged from a molecular model (17) the epitope region of this antibody is located on the surface of PLTP and well exposed. Polyclonal antibodies against recombinant human PLTP (rhPLTP) and apoA-I were raised in New Zealand White rabbits. In brief, 250 µ of purified rhPLTP or apoA-I in PBS suspended in Freund’s complete adjuvant was injected into rabbits subcutaneously. The rabbits subsequently received three booster injections at 2-week intervals. Polyclonal antibodies against rhPLTP and apoA-I were purified from rabbit serum by ammonium sulfate precipitation and Protein A-Sepharose chromatography. The specificity of these antibodies was confirmed by SDS-PAGE and Western blotting (data not shown).

General Procedures—SDS-PAGE was performed by the Laemmli method (18), followed by Coomassie staining or Western blotting and ECL detection (19). Protein concentration was determined by the method of Lowry et al. (20).

Heparin-Sepharose (H-S) Affinity Chromatography (Large Scale)—Affinity chromatography was performed using a 250-ml heparin-Sepharose 6 Fast-Flow column (column dimensions, 4.5 × 12 cm) (Amersham Biosciences) equilibrated with 25 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA. Fresh human plasma (50 ml) was applied and recycled on the column overnight at +4 °C with a flow rate of 3 ml/min. The column was washed with the same buffer, flow rate of 10 ml/min, and thereafter the bound material was eluted with a linear 0–0.5 M NaCl gradient at a flow rate of 5 ml/min; 10-ml fractions were collected and analyzed for PLTP activity and mass.

Heparin-Sepharose Affinity Chromatography (Small Scale)—The active PLTP fractions (high activity (HA)-PLTP) recovered from the 250-ml H-S column were combined and dialyzed against 25 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and then applied to a 5-ml HiTrap heparin column (Amersham Biosciences). The column was washed with the same buffer containing 0.1 mM NaCl, flow rate of 2 ml/min. The bound material was eluted with a linear 0.1–1 M NaCl gradient (flow rate, 0.5 ml/min; fraction size, 2.5 ml). The fractions were analyzed for PLTP activity and mass.

Hydrophobic Chromatography—The inactive PLTP fractions (low activity (LA)-PLTP) recovered from the 250-ml H-S column were combined and adjusted with NaCl to a final concentration of 2 M. The LA-PLTP was applied to a 2 × 5-cm butyl-Sepharose 4 Fast Flow column (Amersham Biosciences) equilibrated with 10 mM Tris-HCl buffer, pH 7.4, containing 2 mM NaCl and 1 mM EDTA at a flow rate of 2 ml/min. The column was washed with 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and eluted with 50% (v/v) ethanol. The fraction size was 4 ml.

Immunoadfinity Chromatography—The monoclonal anti-PLTP-antibody JH66 (21) was coupled to cyanogen bromide-activated Sepharose 4B (17.8 mg of IgG/3 ml of gel) according to the manufacturer’s instructions (Amersham Biosciences). The LA-PLTP fractions from the butyl-Sepharose column were dialyzed against PBS and applied on the anti-PLTP column (1 × 3 cm) equilibrated with PBS. The column was washed in two steps: first with PBS and then with PBS containing 0.2% Tween 20, and 2.5-ml fractions were collected. The material bound to the antibody column was eluted with 0.1 M glycine, pH 2.5, containing 0.2% Tween 20 into two tubes containing 1 M Tris-HCl, pH 8.5, for neutralization. The elution was performed at a flow rate of 0.5 ml/min, and 1-ml fractions were collected.

Hydroxylapatite Chromatography—The HA-PLTP fractions from the small scale H-S chromatography were combined and applied to a hydroxylapatite column (Bio-Gel HTP, Bio-Rad, column dimensions, 1.3 × 2 cm) equilibrated with 1 mM sodium phosphate buffer, pH 6.8, containing 150 mM NaCl, at a flow rate of 0.5 ml/min. Protein bound to the column was eluted with a linear 1–50 mM sodium phosphate gradient followed by 100 mM phosphate, and 1-ml fractions were collected.

Immunoprecipitation of PLTP with Anti-PLTP and Anti-apoA-I Antibodies—Immunoglobulins (100 µg) isolated from anti-rhPLTP, anti-apoA-I, or control rabbit serum were coupled to Protein G-Sepharose (50 µg) (Amersham Biosciences). After the beads were washed, 50 µl of the PLTP samples or 5 µl of serum were added and incubated on ice in a total volume of 500 µl for 18 h. After incubation, the beads were pelleted by centrifugation, and PLTP mass and activity were determined from the supernatants. In addition, immunoprecipitation of the LA-PLTP and HA-PLTP was performed using Dynabeads Protein G magnetic beads (Dynal Biotech ASA, Oslo, Norway) following the instructions of the manufacturer. The buffer used in the experiments was PBS, pH 7.4. Briefly, anti-PLTP mAb JH66, rabbit polyclonal anti-apoA-I, or a non-immune IgG was added to washed Dynabeads (250 µl) and incubated with gentle mixing for 40 min at room temperature, after which the beads were washed two times with 500 µl of PBS. Covalent immobilization of the bound IgG was then carried using dimethyl pimelimidate according to the manufacturer’s instructions. The LA-PLTP and the HA-PLTP samples were added to the beads and incubated at room temperature for 1 h with gentle mixing. The beads were recovered, and the supernatant was used for determinations. The beads were washed three times with 1 ml of PBS and then treated with 0.1 M glycine, pH 2.5, and after neutralization the eluted material was used for analyses.

RESULTS

Separation of LA-PLTP and HA-PLTP—We have demonstrated that PLTP in human plasma exists in two forms, one with low (LA-PLTP) and the other with high specific activity (HA-PLTP), and that these can be separated by size-exclusion chromatography (13). In the present study the two forms of plasma PLTP were separated by H-S affinity chromatography (Fig. 1). When plasma was applied to the H-S column, 93 ± 5% (n = 3) of the PLTP activity and 70 ± 15% (n = 3) of the PLTP mass applied were bound to the matrix. The PLTP mass and activity retained in the column were eluted with a linear 0–0.5 M NaCl gradient. The low activity form of PLTP eluted at a NaCl concentration of 0.15–0.2 M, and the high activity form at 0.3–0.4 M NaCl. The total recovery of PLTP activity in this step was 110 ± 16% (n = 3) and that of PLTP mass was 90 ± 21% (n = 3). The specific activity of the LA-PLTP after H-S affinity chromatography (Fig. 1, fractions 78–81) was 0.04 µmol/h/µg whereas that of the HA-PLTP (fractions 92–118) was 3.5 µmol/h/µg, as compared with 0.36 ± 0.22 µmol/h/µg in whole plasma (15). As this purification step does not allow a complete separation of the two forms of PLTP the specific activities of the combined fractions of LA-PLTP and HA-PLTP must be regarded as tentative. Taken together, the two forms of PLTP, HA-PLTP and LA-PLTP, both bind to heparin and can be efficiently resolved by elution with a linear NaCl gradient.

To verify that the LA- and HA-PLTP resolved by H-S affinity chromatography correspond to those described previously (13), the two fractions were subjected to size-exclusion chromatography (Fig. 2). The elution volumes of the two PLTP popula-
tions (Fig. 2, A and B) were similar to those obtained for LA-PLTP and HA-PLTP when whole plasma was chromatographed under identical conditions (Fig. 2C). LA-PLTP and HA-PLTP eluted at positions corresponding to average molecular masses of about 520 kDa and 160 kDa, respectively. Thus, separation of LA- and HA-PLTP by H-S affinity chromatography does not disturb the integrity of the native PLTP complexes.

Characterization of LA-PLTP—To further enrich the LA-PLTP separated from the HA-PLTP by H-S affinity chromatography, the fractions containing LA-PLTP were subjected to hydrophobic chromatography on butyl-Sepharose (Fig. 3). Of the LA-PLTP mass applied, 100% was retained in the column, and 79% could be eluted with 50% EtOH. More than 90% of the protein eluted was apoA-I. To analyze whether the LA-PLTP complex obtained by this step is of the same size as LA-PLTP isolated from plasma by gel filtration, the complex was subjected to gel filtration. It eluted in a position corresponding to an approximated molecular mass of 520 kDa, indicating that the conditions used in the hydrophobic chromatography step had not dissociated the complex. When gel filtration analysis of this LA-PLTP fraction was performed in the presence of 8 M urea, the PLTP protein eluted in a position corresponding to a molecular mass of 160 kDa (Fig. 4). These data suggest that LA-PLTP is part of a large protein complex that can be dissociated with a high concentration of urea.

The LA-PLTP obtained by hydrophobic chromatography was then subjected to chromatography on an immunoaffinity column prepared from the monoclonal anti-PLTP antibody JH 66 (Fig. 5). Of the LA-PLTP applied, 85% was retained by the column and could be eluted by 0.1 M glycine, pH 2.5, containing 0.2% Tween 20. In this purification step the apparent size of the LA-PLTP complex changed significantly. The LA-PLTP recovered had a molecular mass of 160 kDa as assessed by gel filtration (Fig. 4). Although the size of LA-PLTP after this step was similar to that determined for HA-PLTP (see below), no phospholipid transfer activity could be detected in the eluted fractions (data not shown). Most of the apoA-I did not bind to the affinity matrix. However, a significant amount of apoA-I was detected in the eluted LA-PLTP fractions. In the presence of 8 M urea the apparent mass of the 160-kDa mAb column LA-PLTP complex remained unchanged.

SDS-PAGE analysis of the LA-PLTP fraction obtained by Ab-affinity chromatography and gel filtration (Fig. 6) revealed a protein pattern composed of the 80-kDa PLTP identified by Western blotting, and additional protein bands with the apparent molecular masses of 48, 40, and 28 kDa. Of these, the 28-kDa protein was identified as apoA-I. In the LA-PLTP fraction no apoE could be detected.

Characterization of HA-PLTP—To further enrich the HA-PLTP separated from the LA-PLTP by H-S affinity chromatography, the fractions containing HA-PLTP were subjected to a second H-S affinity chromatography (Fig. 7). Of the PLTP activity applied 73% was recovered when the column was eluted with a linear 0.1–1.0 M NaCl gradient. The apoA-I bound to the matrix eluted immediately before PLTP, whereas the elution position of apoE coincided with that of PLTP. The fractions (Fig. 7, fractions 36–40) containing PLTP activity were then combined and subjected to chromatography on hydroxylapatite (Fig. 8). The column was
developed using a linear 1–50 mM sodium phosphate gradient followed by 100 mM sodium phosphate. In this step 36% of the PLTP activity and 91% of PLTP mass were recovered in fractions 19–23. The fractions contained apoE but no apoA-I. The majority of the bound apoE and apoA-I was recovered in the 100 mM phosphate eluate. When the eluted fractions containing HA-PLTP were subjected to size-exclusion chromatography, PLTP activity and mass eluted in a position corresponding to the size 160 kDa, together with apoE (Fig. 9).

Analysis of the HA-PLTP fraction by SDS-PAGE (Fig. 6) revealed, in addition to the 80-kDa PLTP identified by Western blotting, a strong band in the 110–120-kDa region and three groups of bands in the regions of 65–75, 55–65, and 30–35 kDa. Of these, a 34-kDa band was identified as apoE. The identity and possible association of the other proteins with PLTP are under study.

Immunoprecipitation of PLTP—Previous work has suggested that PLTP is capable of associating with apolipoproteins in vitro (22) and in vivo (23). To investigate the association of PLTP with the major apolipoprotein of HDL, apoA-I, immunoprecipitations of plasma and HA- and LA-PLTP fractions obtained by size-exclusion chromatography were performed. Immunoprecipitation with polyclonal anti-PLTP removed about 95% of the PLTP mass from plasma and from the LA- and HA-PLTP fractions, as well as from recombinant human PLTP included as a control. Similarly, PLTP activity was efficiently removed from plasma and from the HA-PLTP fraction (Fig. 10). Non-immune rabbit IgG did not significantly precipitate PLTP protein nor did it remove PLTP activity from the specimens.

Immunoprecipitation of plasma using anti-apoA-I antibodies resulted in coprecipitation of 80% of the PLTP protein. However, anti-apoA-I precipitation only caused a minor 5% decrease in plasma PLTP activity. Anti-apoA-I, when added to the LA-PLTP fraction, caused coprecipitation of 90% of the PLTP protein, indicating that apoA-I and PLTP form a physical complex. Immunoprecipitation of the HA-PLTP fraction with anti-apoA-I resulted in only a marginal 3% decrease of the PLTP activity, suggesting that the active form of PLTP is not associated with apoA-I-containing particles. Similar results were obtained when the LA-PLTP fraction obtained from the hydroxylapatite column were subjected to immunoprecipitation with the same antibodies (data not shown).

DISCUSSION

Although the knowledge on the structure and in vitro functions of PLTP have substantially increased during recent years (8), the physiological role of PLTP has thus far best been illustrated in studies employing either transgenic animal models or adenovirus-mediated overexpression of PLTP (10, 23–25). These studies clearly demonstrate that PLTP plays an essential role in maintaining HDL levels in the context of lipolysis and participates in the removal of cholesterol from macrophages by generating pre-β-HDL particles. Studies on the physiological role of PLTP have been hampered by the lack of an assay for PLTP mass measurement. The development of enzyme-linked immunosorbent assay assays for PLTP protein measurement (15, 16, 26) is thus of great importance and provides essential tools for detailed understanding of the role of PLTP under different physiological conditions.

We recently reported that PLTP mass and activity in human plasma do not correlate (15) and that there are two forms of PLTP in the circulation, one with very low (LA-PLTP) and the other with high specific activity (HA-PLTP) (13). This finding raises the question of the molecular mechanisms responsible for the distribution of PLTP between the two forms. Such mechanisms are likely to be of great importance in the metabolism of HDL as well as of apoB-containing lipoproteins (28). We and others have previously shown that PLTP activity can be separated from cholesterol ester transfer protein activity using H-S affinity chromatography (11, 27). We now report that H-S affinity chromatography can also be used to separate the two forms of PLTP, HA-PLTP and LA-PLTP. More than 90% of plasma PLTP activity and 70% of the PLTP mass were retained by the heparin column. LA- and HA-PLTP fractions could then be separated by elution with a salt gradient. Less than 5% of the total PLTP mass recovered was present in the HA-PLTP fractions. This provides additional support for the notion that only a small fraction of the plasma PLTP protein is responsible for the PLTP activity detected in human plasma and that it expresses a very high specific activity (13). The two forms of PLTP have different heparin binding affinities. PLTP has been suggested to contain a heparin binding domain (29). Conceivably, the low affinity binding of LA-PLTP to the heparin matrix could be mediated directly by this domain. On the other hand, the higher affinity of HA-PLTP could be due to the contribution.
of other proteins showing affinity for heparin. Alternatively, conformational differences between the two forms of PLTP could cause the difference in heparin binding affinity.

LA-PLTP, when fractionated by size-exclusion chromatography, elutes according to a molecular size of 520 kDa. A similar size of the LA-PLTP is also evident in non-denaturing gradient gel electrophoresis (13). When the LA-PLTP complex was chromatographed in the presence of 8M urea, it dissociated, and the PLTP mass eluted at a position corresponding to a molecular mass of 160 kDa. These data suggest that the low activity form of PLTP is part of a relatively large protein complex that is sensitive to dissociation under chaotropic conditions. The complex could in principle represent a homomultimer of PLTP or a complex of PLTP with other plasma proteins and lipids. Interestingly, the present data provide evidence that PLTP in the low activity complex is physically associated with apoA-I. The observation that HDL isolated by ultracentrifugation contains a large proportion of plasma PLTP mass, up to 40%, but only a marginal portion of plasma PLTP activity (13) is in agreement with the present finding that PLTP associated with apoA-I is of low specific activity. The 520-kDa LA-PLTP complex remained intact during the heparin and butyl-Sepharose chromatography steps, but its size decreased in the immunoaffinity purification. It is possible that binding of LA-PLTP to the specific antibody detached a PLTP apoA-I subcomplex, which may have originally been associated, for example, with HDL-like structures that did not bind to the antibody column.

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In size-exclusion chromatography the catalytically active HA-PLTP has an apparent size of 160 kDa (13). The HA-PLTP eluted in a similar position both in the presence and absence of 8 M urea, suggesting that it might represent a PLTP dimer, PLTP associated with lipids, or a urea-resistant relatively small protein complex. PLTP activity either in plasma or in the isolated HA-PLTP fraction could not be immunoprecipitated with apoA-I. However, HA-PLTP was found to copurify with apoE. In agreement with this finding, it was recently reported that PLTP activity increases significantly during inflammation, and in this situation active PLTP showed a major overlap
with apoE upon analysis by two-dimensional gel electrophoresis (23).

It was recently reported that PLTP plays a role in the secretion of apoB-containing lipoproteins (28). Thus, PLTP may initially be part of the secreted nascent VLDL or related particles. The finding that LA-PLTP but not HA-PLTP is associated with apoA-I allows us to suggest a model in which nascent PLTP enters the circulation as a high specific activity form not associated with apoA-I allowing us to suggest a model in which nascent PLTP enters the circulation as a high specific activity form not associated with apoA-I and in this way be sequestered into the LA-PLTP complex. It becomes associated with apoA-I-containing HDL-like particles. The finding that LA-PLTP but not HA-PLTP is associated with apoA-I-containing HDL-like particles. The finding that LA-PLTP but not HA-PLTP is associated with apoA-I-containing HDL-like particles.

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PLTP activity (% of the control)
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